Biochimica et Biophysica Acta, 467 (1977) 280—289
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BBA 77727

SELECTIVE BINDING OF POLYMYXIN B TO NEGATIVELY CHARGED LIPID MONOLAYERS

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(Received November 29th, 1976)

Summary

It is demonstrated by direct measurement of surface radioactivity that the cationic polypeptide antibiotic polymyxin B is specifically adsorbed to negatively charged lipid monolayers. The latter attracted the following amounts of the biologically active mono-*N*-[^{14}C]acetylpolymyxin B derivative (PX): lipid A from *Proteus mirabilis*, 0.17; phosphatidic acid, 0.12; phosphatidylglycerol and phosphatidylserine, 0.11; dicetylphosphate, 0.107; sulfoquinovosyldiglyceride, 0.104; phosphatidylinositol and cardiolipin, 0.095; and phosphatidylethanolamine, 0.017 μg/cm^2. Adsorption of PX to phosphatidylcholine, monogalactosyldiglyceride and stearylamine was almost or completely zero. Total lipids from *Escherichia coli* adsorbed 0.057 in comparison to 0.051 μg PX/cm^2 of an artificial mixture of phosphatidylethanolamine/phosphatidylglycerol/cardiolipin in the proportions 75 : 25 : 5. The concentration of the surface active PX at the air/water interphase was 0.091 μg/cm^2. These saturation surface concentrations of PX at lipid monolayers were reached at 1 μg/ml bulk concentrations in 2 mM NaCl/1 mM Tris · HCl, pH 7.2. They decreased with decreasing surface charge density of the adsorbing monolayer. In an experiment with cardiolipin/phosphatidylethanolamine mixtures it was shown that two molecules of cardiolipin induced adsorption of one molecule PX giving a 1 : 1 ratio with regard to positive and negative charges. This could be due to a similar charge density of about one charge per 40—50 Å^2 in PX and lipid bilayers composed of phospholipids. The electrostatic PX-lipid interaction was severely inhibited by 10^{-2} and 10^{-1} M Ca^{2+} and Na^+, respectively. It is discussed that the specificity of PX against Gram-negative bacteria is caused by the occurrence of lipid A, phosphatidylglycerol and cardiolipin at the cell surface of these microorganisms.

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Abbreviation: PX, mono-*N*-[^{14}C]acetyl-polymyxin B derivative.

Introduction

The basic polypeptide antibiotic polymyxin B is a clinically important reserve antibiotic which is used to treat severe cases of infections by *Pseudomonas* and certain other Gram-negative bacterial species [1]. It is of interest because of its specific action against Gram-negative bacteria [2]. The target sites in susceptible microorganisms are the cell envelope and the cytoplasmic membrane [2,3]. Experiments with lipid monolayers [4], liposomes [5—7], isolated phospholipids and membranes [8,9], and with polymyxin-resistant bacteria [2,10] suggested that the cationic antibiotic molecule needs anionic phospholipids like cardiolipin or phosphatidylglycerol in order to bind to and destroy biological membranes. In this publication we confirm a stoicheiometric binding of polymyxin B to the negatively charged lipid monolayers.

Materials and Methods

Polymyxin B sulfate (sterile powder) was a generous gift from Pfizer GmbH (Karlsruhe, Germany). Mono-*N*-[^{14}C]acetyl-polymyxin B (59.5 Ci/mol) was synthesized by reaction of 500 μCi [1-^{14}C]acetic anhydride (119 Ci/mol, Amersham Buchler) with 50 mg polymyxin B sulfate dissolved in 2.5 ml aqueous 0.1 M $NaHCO_3$ as previously described [11]. The mixture was left at 0°C for 30 min and made more basic by addition of 5 mg Na_2CO_3. Polymyxin B and its radioactively labelled *N*-acetyl derivatives were extracted with five successive 0.5-ml portions of *n*-butanol. Mono-*N*-[^{14}C]acetyl-polymyxin B was separated from unreacted antibiotic by thin-layer chromatography on cellulose-coated aluminum foils (Merck no. 5552, Darmstadt, Germany) with the solvent system *n*-butanol/pyridine/acetic acid/water (6 : 4 ; 0.3 : 3, v/c). The radioactive antibiotic derivative was eluted from the cellulose with small amounts of 0.14 M aqueous NaCl. It was diluted to a suitable specific activity with non-radioactive mono-*N*-acetyl-polymyxin B which had been prepared by the same method. Phosphatidic acid, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, cardiolipin, phosphatidylethanolamine and phosphatidylcholine were purchased from Lipid Products (Nutfield, England). Their purity was checked by thin-layer chromatography on silica gel [12,13]. Monogalactosyldiglyceride and sulfoquinovosyldiglyceride were extracted and purified to homogeneity from the blue-green bacterium *Anacystis nidulans* (Synechococcus spec.) by R. Bronnenmeier (Department of Microbiology, Technische Universität, München) following a modified procedure of Olsen and Ballou [12]. *Escherichia coli* total lipids were extracted from late log phase cells of *E. coli* B grown in nutrient broth [13]. The lipid A part from *Proteus mirabilis* D52 was prepared from isolated lipopolysaccharide as previously reported [14]. Spectra grade hexane (Fluka, Switzerland) was used without distillation. Tris base was purchased from Sigma (St. Louis, Mo., U.S.A.). Water was doubly distilled over permanganate in a Pyrex still. The experiments were performed in NaCl solutions at different concentrations buffered with 1 mM Tris · HCl to pH 7.2.

The adsorption of polymyxin B was investigated in small stainless steel troughs (25 cm^2 area) at room temperature (20°C). The lipids (about 15 μg

in 5 μl solvent) were spread in excess from hexane solutions after cleaning the aqueous interface. In some cases addition of 10% chloroform to the hexane was necessary to obtain dissolution of the lipids. A condensed monolayer at equilibrium with excess material forming lenses was obtained as previously reported [15]. Assuming an average area of 50 Å^2 per molecule phospholipid and a molecular weight of 800, the amount of applied lipid (0.6 μg/cm^2) constitutes a 2.3-fold excess over that required to form a monolayer. The surface pressure of the monolayers was not determined since a much larger excess of phospholipid (10-fold) did not affect the saturation concentration of polymyxin B indicating that the antibiotic could only adsorb to the surface of the formed microcrystals or lenses but could not penetrate them. The actual surface pressure was close to the collapse pressure.

The radioactive mono-*N*-acetyl-polymyxin B was introduced directly into the solution under the monolayer through plastic tubing. For measuring the adsorption of polymyxin to the air/water interphase the labelled material was injected into the solution without the lipid monolayer present. The adsorption of the labelled polymyxin B to the surface was determined by measuring the surface radioactivity with a gas flow counter equipped with an ultrathin window [16]. The window of the counter was calibrated by comparing the surface radioactivity of dilute [^{14}C]cholesterol with the same amount of labelled material measured in a scintillation counter. The efficiency of the gas flow counter was usually between 7.5 and 8%. The counts originating from the bulk phase were subtracted. The radiation from the bulk was calculated knowing the absorption of β-radiation by water. The calculated values are in agreement with the measured values in absence of adsorption to the surface using $NaH^{14}CO_3$.

Results

N-Acetylation of the pentavalent polymyxin B molecule leads probably to a random labelling of one of the five free γ-amino groups of the α, γ-diaminobutyric acid residues, finally a mixture of five possible isomers. However, the tetravalent *N*-[^{14}C]acetyl-polymyxin B derivatives have identical antibiotic potencies compared to the pentavalent natural compound [11,17] justifying their suitability for biological and biophysical experiments. In the investigated pH range of 7.2, all four or five free amino groups are protonated [18]. The hydrophobic parts of the molecule (methyloctanoic acid, D-phenylalanyl-L-leucyl region) together with the positive charges give the polymyxin B molecule the properties of a surface-active cationic detergent [2]. This surface activity is demonstrated with our system in Fig. 1. Mono-*N*-[^{14}C]acetyl-polymyxin B is rapidly adsorbed to the surface in 2 mM NaCl/1 mM Tris · HCl, pH 7.2, from very low bulk concentrations, the rate of adsorption being presumably diffusion controlled as supported by a linear plot of Γ versus $t^{1/2}$. Essentially the same result is obtained in 0.1 and 1 M NaCl. Assuming a molecular weight of 1280 for the *N*-acetyl derivatives, the maximum amount of 0.091 μg absorbed per cm^2 corresponds to 4.3 $\cdot 10^{13}$ molecules/cm^2 or 232 Å^2 available per molecule polymyxin B.

Before determining the lipid specificity, it was necessary to investigate the time course and concentration dependence of polymyxin B adsorption to a

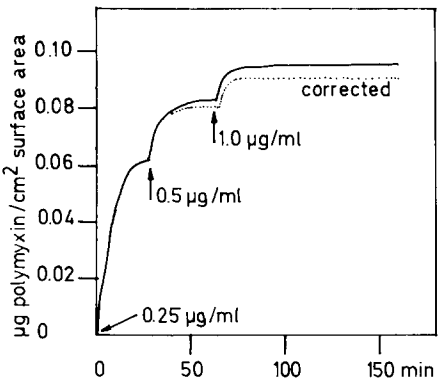

Fig. 1. Time-dependent adsorption of mono-*N*-[^{14}C]acetyl-polymyxin B to the air/water interphase in 2 mM NaCl/1 mM Tris · HCl, pH 7.2, at 20°C. Arrows indicate time of injection of polymyxin into the aqueous phase leading to bulk concentrations given beneath each arrow. The curve corrected for bulk polymyxin is drawn in dashed line.

representative monolayer. We show the results with *E. coli* lipids, however, concomitant data were obtained in principle with the purified acidic lipids. Fig. 2 depicts the time course of binding when a saturating concentration of 1 µg polymyxin B/ml was used. 60% of the maximum amount of adsorbed antibiotic was attached to the surface within 2 min, the process was complete within 16 min. This figure demonstrates also that addition of the positively charged lipid stearylamine to *E. coli* lipids abolishes adsorption of polymyxin B to this monolayer at 25.4 mol% competely and partially at 14.6 mol%. From the latter value it is calculated that a minimum of 21 mol% stearylamine in

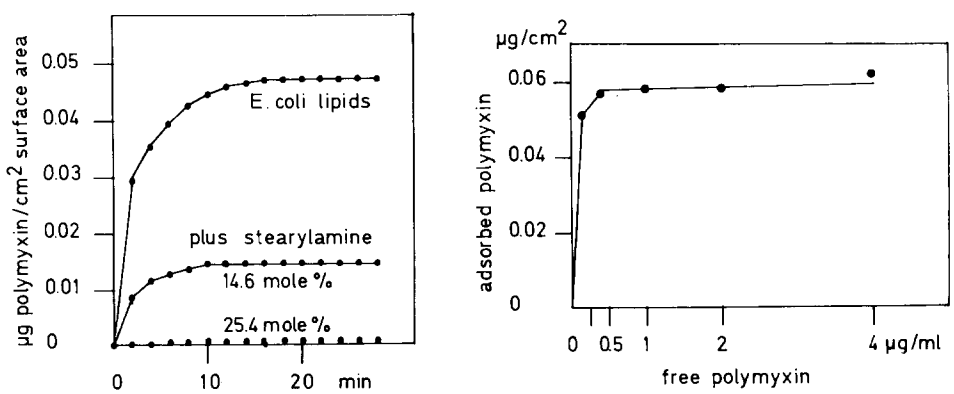

Fig. 2. Time-dependent adsorption of mono-*N*-[^{14}C]acetyl-polymyxin B to monolayers of *E. coli* B total lipids and mixtures thereof with 14.6 and 25.4 mol% stearylamine. These numbers were calculated on the assumption that the *E. coli* lipid fraction is composed by weight of 75 parts phosphatidylethanolamine, 25 parts phosphatidylglycerol and 5 parts cardiolipin. Polymyxin concentration was 1 µg/ml 2 mM NaCl/1 mM Tris · HCl, pH 7.2.

Fig. 3. Adsorption isotherms of mono-*N*-[^{14}C]acetyl-polymyxin B on an *E. coli* B lipid monolayer spread from hexane on 2 mM NaCl/1 mM Tris · HCl, pH 7.2. Surface concentration of labelled polymyxin was measured at equilibrium.

E. coli lipids suffices to make this lipid mixture unattractive for polymyxin B. Fig. 3 shows that an amount of 1 μg of polymyxin B/ml is indeed enough for saturation of the system. It should be noted that 1 μg polymyxin/ml is in the range of the minimum inhibitory concentration for polymyxin-susceptible bacteria [2].

Since the stearylamine experiment described in Fig. 2 clearly suggested the need of negative charges in a lipid monolayer in order to be attractive for the cationic polymyxin B, a final answer on this principle of the polymyxin-lipid interaction should be possible by the use of pure and defined lipids and mixtures thereof. Table I summarizes these results giving unequivocal proof that only the negatively charged lipids like lipid A, phosphatidic acid, phosphatidylglycerol, phosphatidylserine, dicetylphosphate, sulfolipid, phosphatidylinositol, cardiolipin, and to some extent phosphatidylethanolamine induce a surface adsorption of polymyxin B. The neutral glycolipid monogalactosyldiglyceride and the positively charged stearylamine are completely unadsorbing. Adsorption to phosphatidylcholine is very low. In addition, an artificial mixture of pure phosphatidylethanolamine/phosphatidylglycerol/cardiolipin in proportions similar to *E. coli* total lipids yields with 0.051 μg polymyxin/cm^2 surface a value comparing favourably with 0.057 μg/cm^2 in the case of natural *E. coli* lipids. The minimum areas covered by one molecule polymyxin B in the order of 178–224 Å^2 depending on the nature of the lipid monolayer correspond well with

TABLE I

BINDING CAPACITIES OF CONDENSED LIPID MONOLAYERS FOR MONO-*N*-[^{14}C]ACETYL-POLYMYXIN B (PX)

15 μg lipids dissolved in 5 μl hexane were spread onto aqueous 2 mM CaCl/1 mM Tris · HCl, pH 7.2. PX was injected into the aqueous phase to a bulk concentration of 1 μg/ml. The surface concentration of PX was determined after obtaining equilibrium by the use of a gas flow counter. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

Lipid	Surface concentration of labelled PX (μg/cm^2)	Available area per PX molecule (Å^2)
Lipid A from *P. mirabilis*	0.170	*
Phosphatidic acid	0.170	*
Phosphatidic acid (7.5 μg)	0.120	170
Phosphatidylglycerol	0.110	194
Phosphatidylserine	0.110	194
Dicetylphosphate	0.107	199
Sulfoquinovosyldiglyceride	0.104	205
Phosphatidylinositol	0.095	224
Cardiolipin	0.095	224
Phosphatidylethanolamine	0.017	1250
Phosphatidylcholine	0.006	3540
Monogalactosyldiglyceride	0.000	—
Stearylamine	0.000	—
PE/PG/CL, 75 : 25 : 5	0.051	417
E. coli total lipids	0.057	373
Air/water interphase	0.091	232

* Assuming that 0.1 μg PX/cm^2 surface corresponds to 4.7 · 10^{13} molecules, adsorption of 0.17 μg PX per cm^2 leaves only 125 Å^2 per adsorbed PX molecule, an area smaller than the presumed size of PX at the air/water interphase. This could be due to a different orientation and/or conformation of the PX molecule.

the size of polymyxin E (about 180 Å^2) determined many years ago by Few and Schulman [19]. However, the conformation of free and adsorbed polymyxin B is still not known.

In conclusion, the saturation surface concentration of polymyxin B seems to decrease with decreasing surface charge densities of the adsorbing lipid monolayer. If this holds true, there should be a stoicheiometric relation between the number of negative charges in the lipid monolayer, as suggested by the stearylamine experiment, and the number of positive charges from polymyxin B adsorbed to the monolayer. This phenomenon is shown in Fig. 4 and 5. In cardiolipin/phosphatidylethanolamine mixtures, the amount of polymyxin B adsorbed to the surface is linearly dependent on the area contributed to by increasing amounts of cardiolipin (Fig. 4). Fig. 5 correlates the number of tetravalent mono-*N*-[^{14}C]acetyl-polymyxin B molecules adsorbed with that of the divalent cardiolipin per unit area. The observed experimental points coincide with a straight line drawn for a theoretical 1 : 2 relation. This means a 1 to 1 ratio with regard to positive and negative charges. These exists biological [19] and biochemical [8] evidence for stoicheiometric interactions between polymyxin B and negatively charged lipids. In the present work, we have confirmed this assertion by a quantitative physicochemical method. The dependence of polymyxin binding to monolayers of *E. coli* lipids on the ionic strength of the aqueous phase further emphasizes the importance of ionic interaction. As ex-

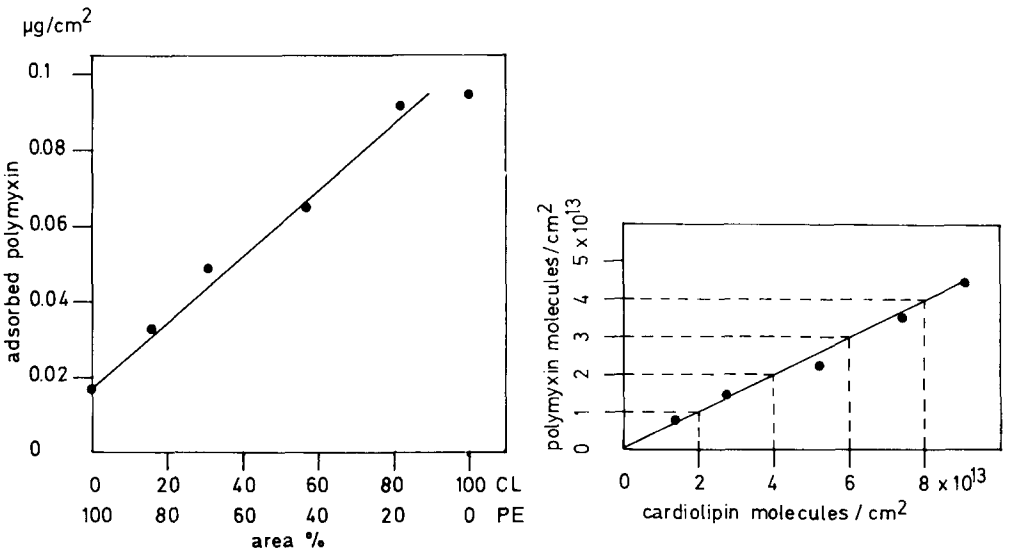

Fig. 4. Cardiolipin-dependent adsorption of mono-*N*-[^{14}C]acetyl-polymyxin B to mixed monolayers of phosphatidylethanolamine and cardiolipin. The area covered by cardiolipin was calculated assuming a molecular weight of 750 and a surface of 50 Å^2 for one molecule phosphatidylethanolamine, and a molecular weight of 1325 and a surface of 110 Å^2 for one molecule cardiolipin [21,22]. The determination was performed with 1 µg polymyxin B/ml 2 mM NaCl/1 mM Tris · HCl, pH 7.2.

Fig. 5. Linear dependence of the number of polymyxin molecules absorbed on the number of cardiolipin molecules present per unit area as calculated from the data shown in Fig. 4. The binding due to phosphatidylethanolamine has been subtracted.

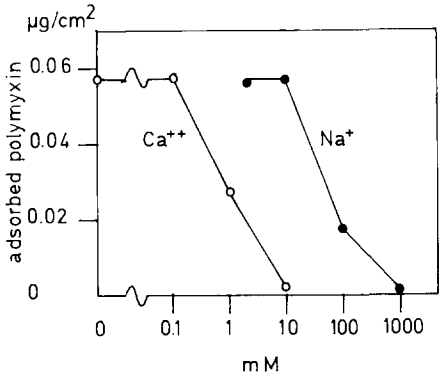

Fig. 6. Surface concentration of mono-*N*-[^{14}C]acetyl-polymyxin B adsorbed to *E. coli* B lipid monolayers as a function of NaCl and $CaCl_2$ concentration in 1 mM Tris · HCl, pH 7.2. 1 µg polymyxin B/ml was applied in the aqueous phase and surface concentration measured after obtaining equilibrium.

pected, high concentrations of Na^+ or Ca^{2+} inhibit the adsorption of polymyxin B to the surface. Nevertheless, polymyxin B has been shown to be active against liposomes prepared from *E. coli* lipids at 1 M NaCl [20]. Obviously, binding of polymyxin at a few binding sites is sufficient to cause membrane leakage in liposomes and living cells.

The low adsorption of polymyxin B to monolayers of phosphatidylethanolamine is due to a slight negative net charge of this zwitter ion at pH 7.2. Lowering the pH to 3.5 completely abolished this effect, whereas an increase to pH 9 led to an approximate increase of polymyxin adsorption by 50%. The quantitative evaluation of these data at high pH values is ambiguous because of increasing simultaneous deprotonation of phosphatidylethanolamine and poly-

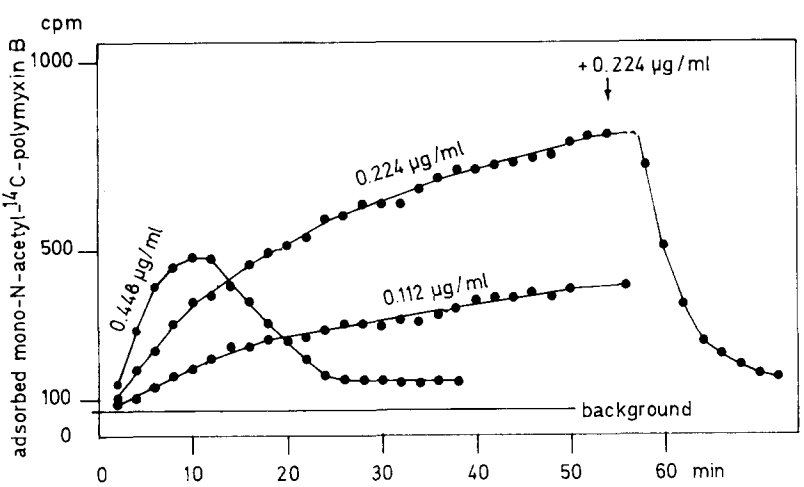

Fig. 7. Adsorption of mono-*N*-[^{14}C]acetyl-polymyxin B (58.5 Ci/mol, 1 part) in a mixture with natural polymyxin B (19 parts) to phosphatidylinositol monolayer from 2 mM NaCl/1 mM Tris · HCl, pH 7.2.

myxin amino groups. Uncharged, completely *N*-acetylated polymyxin B does not bind to lipid A [14].

An interesting phenomenon was observed when tetravalent radioactive mono-*N*-[^{14}C]acetyl-polymyxin B was diluted with a 20-fold excess of non-radioactive pentavalent natural antibiotic. As shown in Fig. 7, a rapid adsorption of the radioactive derivative was followed by a desorption of radioactive material. With increasing total concentration of polymyxins, the process was accelerated. Addition of excess polymyxin to an equilibrated polymyxin-monolayer complex induced a rapid desorption of mono-*N*-[^{14}C]acetyl-polymyxin B. This behaviour was found with all investigated acidic lipids including the sulfolipid. Primarily adsorbed tetravalent *N*-acetyl-polymyxin is obviously replaced by non-radioactive pentavalent antibiotic. This competition indicates that the modified molecule is binding to the same site as the natural antibiotic. A detailed mathematical treatment of this kinetic will be published elsewhere (Miller et al., in preparation).

Discussion

Adsorption of polymyxin B to lipid monolayers has been shown in this publication to be due to a presumably diffusion-controlled electrostatic interaction as long as adsorption forces maintain a practically zero concentration in the bulk layer adjacent to the surface. This is documented mainly by the following observations: (1) Polymyxin is only adsorbed to monolayers containing negatively charged lipids, and (2) this adsorption is quantitatively related with the charge density of the lipid layers. Dilution of cardiolipin monolayers with phosphatidylethanolamine or of *E. coli* total lipids with stearylamine leads to a stoicheiometric reduction of polymyxin adsorption. The cardiolipin experiment demonstrated that every two cardiolipin molecules irrespective of their absolute concentration in the mixed cardiolipin/phosphatidylethanolamine monolayer induced the adsorption of one molecule of the tetravalent mono-*N*-[^{14}C]acetyl-polymyxin B (see Fig. 5). On the basis of the involved positive and negative charges of polymyxin and cardiolipin, respectively, this yields a one to one ratio. Assuming a surface area of about 200 Å^2 for one polymyxin molecule (see Table I) and 220 Å^2 for two cardiolipin molecules [22] we find an almost perfect steric matching of the antibiotic molecules with its receptor molecules. Therefore, the similarity of the charge density in molecular terms with 40—50 Å^2 available per positive charge in polymyxin and per negative charge in phospholipid monolayers must be the reason for the high affinity of polymyxin for negatively charged lipid layers.

Whereas our data clearly prove that polymyxin is adsorbed to individual acidic lipid species in lipid monolayers, this has not been possible in lipid bilayer systems like liposomes. However, a polymyxin-induced leakiness of liposomes for entrapped glucose has only been demonstrated in liposomes containing negatively charged phospholipids [6]. Since acidic lipid monolayers were saturated with polymyxin B at a bulk concentration of 1 μg/ml or about 10^{-6} M, we believe that the interaction of polymyxin B with phosphatidylcholine at extremely high concentrations of 300 mg/ml [7] is not necessarily relevant for the selective action of the antibiotic against negatively charged liposomes and

Gram-negative bacteria. We must postulate from the described mono- and bilayer experiments that Gram-negative bacteria are susceptible to polymyxin B if they contain negatively charged lipids at the cell surface. Indeed, phosphatidylglycerol, cardiolipin and the acidic lipid A part of lipopolysaccharide are major components of the outer membrane in Gram-negative bacteria [23]. Also, fusion of polymyxin-resistant *Acholeplasma laidlawii* B with phosphatidylglycerol or cardiolipin vesicles induced polymyxin susceptibility [10]. However, the most convincing evidence for acidic lipids as polymyxin receptor molecules in the membranes of Gram-negative bacteria [9] would be a living system which allows a managable change in membrane lipid composition from acidic to basic lipids. Minnikin and Abdolrahimzadeh [24] found that limitation of inorganic phosphorus in the growth medium led to a replacement of phosphatidylethanolamine, cardiolipin and phosphatidylglycerol by an ornithine-amide lipid in *Pseudomonas fluorescens*, a highly polymyxin-susceptible bacterium. Dorrer [25] could show that this switch in lipid composition produces a complete polymyxin resistance in *P. fluorescens* (Dorrer, E. and Teuber, M., manuscript in preparation). However, the mechanism how adsorbed polymyxin induces membrane leakage in liposomes and microorganisms remains unknown. The fact that polymyxin B can be extracted into chloroform if it contains acidic phospholipids [8] led us to speculate that hydrophobic interactions might be involved in addition to electrostatic forces. Electrostatic attraction of the antibiotic must be the necessary first step inducing further reactions like penetration into or through the membrane. The experimental approach of this publication was not designated to solve this problem since the used monolayers differed in two important properties from biological membranes: (1). They did not contain membrane proteins, and (2) they have a planar surface in contrast to a curved surface in liposomes and living cells.

Acknowledgements

The investigations were made possible by a short term fellowship of the European Molecular Biology Organisation granted to one of us (M.T.). We thank Dr. Diana Bach for stimulating discussions.

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