Titration calorimetric studies to elucidate the specificity of the interactions of polymyxin B with lipopolysaccharides and lipid A

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Lipopolysaccharide (LPS), the major cell wall constituent of Gram-negative bacteria, evokes a multitude of biological effects in mammals including pyrogenicity and toxic shock syndrome. Polymyxin B (PmB), a polycationic cyclic peptide, is known to neutralize most of its activities. The nature of the interaction of PmB with LPS and lipid A was investigated by isothermal titration calorimetry. PmB binds to LPS as well as lipid A stoichiometrically and non-co-operatively with micromolar affinity. These interactions are driven primarily by a favourable change in entropy (ΔS) and are endothermic in nature. These positive changes in enthalpies decrease with increasing temperature, yielding a heat capacity change, ΔC_p , of

–2385 J·mol^{–1}·degree^{–1} for PmB–LPS interactions while the binding of PmB to lipid A displays a ΔC_p of –2259 J·mol^{–1}·degree^{–1}. The negative heat capacity changes provide strong evidence for the role of hydrophobic interactions as the driving force for the association of PmB with LPS and lipid A. A correlation of the energetics of these interactions with analyses of the molecular models of PmB suggests that a cluster of solvent-exposed non-polar amino acid side-chains that line one surface of the molecule, together with a ring of positively charged residues on its other surface, are responsible for its strong and stoichiometric binding to LPS.

INTRODUCTION

Recognition and interaction between microbial and eukaryotic systems is mediated by specific molecules unique to each organism. Lipopolysaccharide (LPS), a major cell wall constituent of Gram-negative bacteria, is one such example of a molecule. It is made up of a carbohydrate chain of variable size and sequence that is covalently attached to lipid A (an acylated phosphodisaccharide unit). Biological effects of LPS emanate from its lipid A moiety which constitutes its conserved hydrophobic region [1]. LPS plays an important role in the pathophysiology of Gram-negative sepsis and the endotoxic shock thus brought about is fatal in as many as 60% of the victims [2]. Other pleiotropic effects of LPS include B-cell mitogenesis, activation of complement and of viruses in monocytes and macrophages, release of cytokines, mimicry of symptoms of acute-graft versus host disease, pyrogenicity in mammals and triggering of the defence and clotting cascade in the horseshoe crab [1,3].

Polymyxin B (PmB), a cyclic cationic peptide antibiotic, neutralizes many of the biological activities of LPS by binding to it [4]. Though the biological effects of LPS that are neutralized by PmB are well characterized, the precise mode of its binding to LPS and the structural features involved therein are unknown. Its use in treating sepsis, etc. is severely compromised due to its high toxicity [5].

As probing the nature and magnitudes of the forces involved in macromolecule–ligand interactions is fundamental to the design of molecules that bind to specific biological targets and inhibit their function in disease processes, we have undertaken titration calorimetric studies on the binding of PmB with lipid A and LPS. These studies reveal that PmB displays a large negative ΔC_p for binding to lipid A and LPS, indicating that non-polar forces are primarily responsible for this recognition. This in turn suggests that the segregation of non-polar and charged polar

groups on the opposite faces of the PmB molecule are responsible for its observed biological activity.

EXPERIMENTAL

Materials

LPS from *Escherichia coli* strains 011:B4 and 055:B5, obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), were repurified according to the phenol–chloroform–petroleum ether extraction method of Galanos et al. [6] and treated with proteinase K, DNase I and bovine pancreatic ribonuclease. Low-molecular-mass impurities were removed by gel filtration on a Sephadex G-200 column equilibrated and eluted with water. Samples were subsequently lyophilized. LPS preparations thus obtained contained less than 0.5% protein and 1% nucleic acid. LPS samples were also freed of bivalent cations by dialysis against 10 mM EDTA in Tris/HCl buffer, pH 7.0, for 3 days to remove bivalent cations as described by Shands and Chun [7]. They were subsequently dialysed against deionized water and lyophilized. LPS preparations thus prepared contained less than 0.009% magnesium and 0.01% calcium by weight. LPS from *Salmonella typhimurium* and *Salmonella enteridis* were the products of BioCarb Chemicals, Sweden. They were also repurified using the above procedures and had similar purities to those of *E. coli* LPS molecules. LPS (Re) from *Salmonella minnesota* Re 595 and diphosphoryl lipid A derived from it were obtained from Ribi Immunochem. U.S.A. Monophosphoryl lipid A (heptacyl) derived from *E. coli* and the PmB were obtained from Sigma. Diphosphoryl lipid A from the deep rough strain of *E. coli* K-12 D31m4 [8] was obtained from List Biological, U.S.A. and demetallized as described above. Tris(hydroxymethyl)aminomethane (THAM) was obtained from the National Institutes of Standards and Technology, Gaithersburg, U.S.A. Bio-Gel P4 was from Bio-Rad, Richmond,

Abbreviations used: LPS, lipopolysaccharide; PmB, polymyxin B; THAM, Tris(hydroxymethyl)aminomethane; DAB, α,τ -diaminobutyric acid.

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CA, U.S.A. All other reagents and solvents used were of the highest purity available.

Molecular-mass determination of LPS

Weight average molecular masses of the LPS molecules were determined as described by Shands and Chun [7]. Briefly, 0.75 mg/ml of LPS dissolved in 0.3% deoxycholate in 0.1 M Tris/HCl buffer, pH 8.0, was subjected to equilibrium sedimentation in a Beckman Model E analytical centrifuge at 24630 rev./min. The data were analysed according to the method of Yphantis [9]. The mass for LPS–deoxycholate complex was calculated from the slope of the plots of $\ln C$ (concentration) displacement versus radial distance squared (X^2) during the equilibrium sedimentation using eqn. (1).

$$M_r = \frac{2RT}{(1 - \bar{V}_p)\omega^2} \cdot \frac{d \ln C}{d(X^2)} \quad (1)$$

where $(1 - \bar{V}_p)$ is the buoyancy factor and ω is the angular velocity. A value of 0.68 for the partial specific volume ($\bar{V}$) of LPS was used [7]. Molecular masses of anhydrous LPS molecules were estimated by correcting for the amount of deoxycholate bound [7].

Chemical modifications of PmB

Reductive methylation

PmB was reductively methylated using the method described in [10]. To 10 mg of PmB in borate buffer (pH 9.0) at 0 °C sodium borohydride (0.5 mg) was added, followed by addition of six 5 μ l aliquots of 3.5% formaldehyde at 10 min intervals. The reaction was repeated twice after which PmB was separated from reagents by gel filtration on a Bio-Gel P4 (1.0 cm $\times$ 30 cm) column equilibrated and eluted with water. Fractions (0.5 ml) were monitored at 235 nm.

Acetylation

Acetylation of PmB was performed according to the method of Fraenkel-Conrat [11]. Acetylated PmB was separated at 10, 20, 30, 45 and 60 min from the reagents by gel filtration as described above.

Formylation

Formylation of PmB was carried out as described by Srinivasa and Ramachandran [12]. Subsequent to gel filtration, partially and fully formylated derivatives of PmB were separated from the non-formylated derivatives using paper electrophoresis as described in [12].

Quantification of amino groups

Amino groups in PmB before and after modification were quantified according to the method of Habeeb [13] using 0.1% picrylsulphonic acid. PmB derivatives with all of their amino groups modified were detected using Rydon's reagent [14].

Quantification of LPS/lipid A and PmB

Subsequent to sonication, all the LPS and lipid A samples were subjected to a *Limulus* amoebocyte lysate assay utilizing pyrogen-free water according to the method of Yin et al. [15] prior to titration calorimetry. Endotoxin from Endosafe (U.S.A.) was

used as a standard for the construction of the calibration curve. PmB was quantified by its molar absorbance coefficient (150 at 254 nm) measured in this study.

Isothermal titration calorimetry

LPS or lipid A (dissolved in 50 mM sodium phosphate buffer, pH 6.8) containing equimolar quantities of triethylamine with respect to the anionic groups in the former, was vortexed vigorously for 15 min and then heated at 60 °C for 10 min. Samples were then sonicated for 5 min prior to use.

Titration was performed using the OMEGA high-sensitivity microcalorimeter manufactured by MicroCal Inc. (Northampton, MA, U.S.A.). A detailed description of the design and operation of this instrument has been provided previously [16]. The instrument was calibrated by (i) measuring the area under a test electrical heat pulse and comparing it with heat input, and (ii) measuring the heat of a standard chemical reaction. For the latter, the heat of protonation of THAM by HCl was measured [17]. The two calibration methods were in excellent agreement. For measurement of heat exchanges accompanying the binding of LPS or lipid A to PmB, the former was loaded into the sample cell of the calorimeter (volume = 1.3466 ml) and the reference cell was filled with water containing 0.05% sodium azide. In all cases, the value C , defined as the product of binding constant K and the concentration of binding sites, was in the range 10–25. Next, PmB, or its derivatives in the same buffer, were placed in a 100 μ l syringe at a concentration at least 20–25-fold higher than that of the LPS.

The system was allowed to equilibrate and a stable baseline recorded before initiating an automated titration. A typical titration sequence involved 26 injections at 3 min intervals of 3.6 μ l aliquots of PmB into the sample cell. Throughout the titration, the cell was stirred continuously at 400 rev./min. The results presented correspond to experimental traces of heat absorbed versus time (injection number). The molecular mass of LPS was taken as 20000 Da. The molecular mass of the diphosphoryl lipid A from the deep rough mutant of *E. coli* K-12 D31m4 was taken as 2034 Da [8] while that of the monophosphoryl form (namely from *E. coli* K-12 obtained from Sigma) was taken as 1954 Da. The molecular mass of *S. minnesota* Re 595 LPS was taken as 2610 Da [18]. Titration of LPS and lipid A even with large aliquots (10–250 μ l) of buffer alone did not produce any kind of heat exchanges, indicating that they were not undergoing any change(s) in the state of their aggregation. Prior to analysis the measurements were corrected for the heat of dilution of the PmB, acylated PmB or collistin by performing separate titrations against buffer. These heats of dilution were extremely small. The resulting corrected data were evaluated to determine the binding stoichiometry (n), association constant (K_b) and enthalpy change (ΔH_b) by non-linear least-square analysis in terms of eqn. (1) [16,19,20]. Using this equation the total heat, Q_t , was then fitted, via a non-linear least-square minimization method, to the total titrant concentration, X_t :

$$Q_t = nM_t \Delta H_b V \{ (1 + X_t/nM_t + 1/nK_b M_t) - [(1 + X_t/nM_t + 1/nK_b M_t)^2 - 4X_t/nM_t]^{1/2} \} / 2 \quad (2)$$

where n is the number of sites, M_t is the LPS monomer concentration, V is the cell volume, K_b is the binding constant and ΔH_b is the binding enthalpy. The expression for the i^{th} injection, Q_i is then given by [19].

$$Q_i = Q_t + (dV_i/2V)(Q_i + Q_{i-1} - Q_{i-1}) \quad (3)$$

where dV_i is the volume of titrant added to the solution in the calorimeter cell.

Figure 1 Calorimetric titration for the binding of PmB to LPS (*E. coli* 055:B5)

Enthalpies were determined by titration of 2.5 mM PmB into 0.05 mM LPS in 50 mM sodium phosphate buffer (pH 6.8) at 19.4 °C in an Omega Titration Calorimeter (MicroCal Northampton, MA, U.S.A.). LPS solution was prepared as described in the text and its molecular mass was taken as 20 000 Da. The cell was thermostatically controlled to ± 0.1 °C with a circulating water bath. (A) Twenty-six 3.6 μ l injections of PmB were made into LPS and the heat exchange measured. Injections occurred over 5 s at 3 min intervals. Incremental heats of binding in relation to the injection number are shown in (B). The data were fitted by the non-linear least-squares minimization method [16] subsequent to the correction for the heats of dilution for PmB, which were very small. No turbidity was observed at the end of these reactions under the experimental conditions reported. The structure of PmB is also shown in (A). X corresponds to the acyl (6-methyl-heptanoyl/octanoyl) group attached to the N-terminus of the molecule and DAB to the α , τ -diaminobutyric acid.

ΔG_b° and ΔS_b° were calculated using the fundamental equations of thermodynamics (eqns. 4 and 5).

$$\Delta G_b^\circ = -RT \ln K_b \quad (4)$$

$$\Delta S_b^\circ = (\Delta H - \Delta G_b^\circ)/T \quad (5)$$

RESULTS AND DISCUSSION

Computer-controlled titration calorimetry, as described by Wiseman et al. [16], is a rapid and accurate method for characterizing biological binding reactions. Microcalorimetry yields direct estimates of the enthalpy of binding, as well as the binding constants. Additionally, calorimetry is the only experimental technique that provides a direct estimate of ΔC_p .

The titration calorimetry results in Figure 1 show that the association of PmB to LPS from *E. coli* 055 B5 occurs with an uptake of heat. An analysis of these results shown in Figure 1(B) yielded a positive enthalpy change of $\Delta H_b = 39$ kJ·mol⁻¹, an equilibrium association constant of 1.2×10^6 M⁻¹ at 14 °C and a stoichiometry of 0.97 for PmB bound/LPS.

Previous studies utilizing indirect approaches such as fluorescence spectroscopy and qualitative methods such as precipitation analyses have revealed various stoichiometries ranging from 1 to 3 molecules of PmB for binding to a molecule of LPS [21,22]. Titration calorimetric measurements which allow not only the evaluation of the thermodynamic parameters for the binding process but also exact stoichiometries involved therein show that under a variety of experimental conditions the association of PmB with LPS or lipid A has a stoichiometry close to 1. In this study we have used the molecular mass of the LPS monomer evaluated by equilibrium sedimentation. Equilibrium

Figure 2 Energetics of PmB–endotoxin interactions

Temperature dependence of the enthalpy change for the binding of PmB to LPS (A) and lipid A (B). The linear dependence of enthalpy with temperature provides a heat capacity change, ΔC_p , of -2385 J·mol⁻¹·degree⁻¹ for the association of LPS (A) and -2259 J·mol⁻¹·degree⁻¹ for lipid A (B) to PmB.

sedimentation studies revealed that the molecular masses of LPS monomers from various sources, in deoxycholate, were quite close to 20 000 Da. Molecular masses thus determined from the average of two experiments were as follows: *E. coli* 011:B4 LPS, 19 800 Da; *E. coli* 055:B5 LPS, 20 500 Da; *S. typhimurium* LPS, 21 000 Da; and *S. enteritidis* LPS, 19 500 Da. These values are in agreement with values reported previously in the literature [7,23,24]. In this context observations of Shands and Chun [7] are worthy of reiteration. Although one might expect the LPS of different bacteria to have different chemical structures, it appears that the size of the LPS monomer may be relatively uniform among the members of enterobacteriaceae. Consequently, a value of 20 000 Da for the LPS monomers used in these studies appears to be justifiable. A value of $n = 1$ for binding of PmB to a variety of LPS molecules is also consistent with this fact. Usage of molecular-mass values which differed by 10 % from 20 000 Da yielded values similar to those reported (in other words they were within ± 5 % of reported values). Moreover, a value of 20 000 Da for the molecular mass of LPS monomer has also been used in several recent studies [25].

LPS molecules are known to have a tendency to undergo aggregation. However, their conversion into triethylamine salts used in this study prevents their aggregation for extended periods of times, i.e. 50–60 h [26], a period much larger than that required for the titration calorimetric experiments; and indeed at the end of experiments all solutions were found to be clear, indicating absence of any contribution of aggregation effects to the heat exchanges observed. (LPS and lipid A samples prepared without treatment with triethylamine gave thermodynamic parameters identical to those in its presence for binding to PmB,

Table 1 Thermodynamic parameters for lipid A–PmB and LPS–PmB interactions

The errors in the determination of K_b , n and ΔH_b were 6, 4.8 and 3.3% respectively. In the experiments with liposomes the errors for K_b , n and ΔH_b were 9.7, 7.8 and 6.4% respectively. Number of experiments was four unless otherwise stated. LPS or lipid A (10–50 μ M) in the 1.3466 ml cell were titrated with 0.4 to 2.5 mM of PmB in a 100 μ l syringe. Actual amounts of LPS/lipid A used in the cell were dictated by the instrument's sensitivity and the amount of heat absorbed.

Lipid A or LPS	T (°C)	n	K_b (10^6 M $^{-1}$)	ΔH_b kJ·mol $^{-1}$	$-\Delta G_b^\circ$ kJ·mol $^{-1}$	ΔS_b J·mol $^{-1}$ ·K $^{-1}$
<i>E. coli</i> 011:B4 LPS	5.7	1.02	2.34	68	34	365
	12.2	0.98	4.32	53	36	314
	20.8	0.96	7.21	32	39	239
	24.9	1.02	8.53	22	40	206
<i>E. coli</i> 055:B5 LPS	19.4	0.97	1.20	24	34	198
	24.9	1.05	3.25	12	37	164
<i>S. typhimurium</i> LPS	24.9	1.00	1.23	51	35	288
<i>S. enteritidis</i> LPS	14.7	0.99	2.13	90	35	432
	25.0	1.04	5.22	64	38	343
LPS <i>S. minnesota</i> Re 595 liposomes	24.8	0.93	8.22	36	39	253
<i>S. minnesota</i> Re 595 LPS	14.0	0.96	2.70	68	35	359
	24.9	1.03	8.18	42	39	274
	30.7	1.03	19.51	27	42	229
Lipid A, <i>E. coli</i> K-12 D31m4	10.0	1.01	4.32	74	36	387
	14.2	1.02	5.4	64	37	351
	24.9	1.00	13.51	41	41	273
	30.7	1.03	19.51	27	42	229
Lipid A, diphosphoryl	10.2	1.06	3.93	67	36	361
<i>S. minnesota</i> Re 595	30.0	1.02	18.90	21	42	209
Lipid A, monophosphoryl	10.4	0.95	3.03	68	35	363
<i>E. coli</i> K-12	29.7	1.04	15.30	23	42	159
Lipid A, diphosphoryl	24.9	0.90	11.72	41	40	274
<i>S. minnesota</i> Re 595 in liposomes						

^a For liposomal experiments 3% of LPS or lipid A were incorporated in dipalmitoyl phosphatidylcholine unilamellar liposomes. Liposomes containing about 36 μ M of LPS or lipid A were titrated with 1.2 mM of PmB by injecting 21 μ l aliquots of the latter at 3 min intervals. About 66% of LPS or lipid A incorporated in these liposomes was taken as accessible on the outer leaflet of these vesicles [53].

^b Values from an average of three experiments only.

indicating that this counterion did not alter the interaction in any detectable manner. However, in the absence of triethylamine slight turbidity was observed around 45–60 min which subsequently made the baselines relatively noisy.) Monotonic decrease in the heat absorbed in the reaction also discounts the possibility of the occurrence of any other event that we are not monitoring but which contributes to the binding event in any significant manner. Observation of aggregation in the PmB–LPS interactions by Rustici et al. [22] in their studies is not surprising as their experiments were conducted with LPS preparations devoid of treatment with triethylamine. Moreover in their experiments LPS molecules were incubated with PmB for prolonged periods of time.

The endothermic binding of PmB to a variety of LPS molecules and lipid A yielded values of ΔH_b which ranged from 16 kJ/mol to 90 kJ/mol, while the values of ΔG_b° ranged from –35 kJ/mol to –42 kJ/mol at 24.9 °C. Thus the binding of PmB to LPS and lipid A is entropy driven. This suggests that hydrophobic interactions play a predominant role in the stabilization of PmB–LPS (or lipid A) complex [27–29]. The role of hydrophobic interactions was probed further by performing the experiments as a function of temperature. As can be seen in Figure 2, the ΔH for the binding of PmB to LPS or lipid A decreased with increasing temperature. The temperature dependence of the change in enthalpy is described well with a linear regression model, assuming a constant ΔC_p in the temperature range of the experiments. These data thus yielded a value for ΔC_p of –2385 and –2259 J·mol $^{-1}$ ·degree $^{-1}$ for the binding of PmB to LPS and lipid A respectively. The negative ΔC_p values again underscore the primacy of hydrophobic interactions in the recognition of LPS or lipid A by PmB [30]. If the correlation

between ΔC_p and the water-accessible non-polar surface area is used, then approx. 1800 Å 2 would be buried when PmB binds to LPS or lipid A [31].

Likewise, collistin, an analogue of PmB [31], shows a positive change in enthalpy and entropy ($\Delta G_b^\circ = -30$ kJ/mol, $\Delta H_b = 19$ kJ/mol, for the *E. coli* 055:B5 LPS at 24 °C) with an n value of 1.04 for binding, suggesting that it also recognizes LPS in a similar fashion. A lower K_b value for collistin as compared with PmB suggests that the replacement of D-Phe by D-Leu at the sixth position of the latter antibiotic diminishes the activity of the resulting compound. This is also consistent with the spectrum of their anti-bacterial activities [21].

An examination of the K_b values as a function of temperature also confirms the role of hydrophobic forces in these interactions. Le Chatelier's principle, as expressed in the fundamental van't Hoff equation (eqn. 6), which is also valid for reaction with non-zero ΔC_p , implies that for an endothermic binding process the association constant should increase with temperature.

$$d(\ln K_b)/dT = \Delta H_b/RT^2 \quad (6)$$

The K_b values indeed show an increase with increasing temperature (Table 1). Moreover, the temperature dependence of the association of PmB with LPS or lipid A, estimated from the relationship between the heat capacity change, K_b and ΔH_b at a reference temperature from eqn. (7), should approximate to experimental values if the assumed reaction faithfully describes the energetics of the system [33,34].

$$K_b = K_{bz} \{ \exp[\Delta H^\circ/R(1/T - 1/T_o)] + [\Delta C_p T_o/R(1/T - 1/T_o)] + [\Delta C_p/R \ln(T/T_o)] \} \quad (7)$$

Figure 3 Stability curve for the binding of PmB with endotoxin

Stability curves for the association of PmB with LPS (A) and lipid A (B) as determined using eqn. (7) described in the text.

where ΔH° and K_{bz} denote values at a reference temperature, and the determined values of ΔC_p of -2259 and $-2385 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{degree}^{-1}$ for lipid A and LPS, respectively, were used for these calculations. As can be seen from Figure 3 this assumption is borne out.

The thermodynamics of the association of LPS with PmB show that the changes in enthalpy and entropy upon binding are compensatory in nature. This 'compensatory' effect is displayed in Figure 4 where ΔH_b values for all the LPS molecules and lipid A are plotted as a function of $T\Delta S_b$ at 298 K ($\pm 0.1 \text{ K}$). The slope of the plot is 1.006 . Enthalpy–entropy compensation has been observed in many protein–ligand interactions where the experimental conditions are fixed and the ligand structure is varied (congener series) [35]. Observation of enthalpy–entropy compensation with values of slope close to one suggests that PmB recognizes lipid A and a variety of LPS molecules in a similar fashion. As the mode of the presentation of LPS and lipid A incorporated in liposomes and free in solution are very different, the similarity of the thermodynamic parameters for their association with PmB together with the enthalpy–entropy compensation underscores the fact that the mode of their presentation and/or aggregation does not appreciably alter their energetics of association with this cyclic peptide.

Enthalpy–entropy compensation could also arise due to subtle solvent shifts at the interacting interfaces of these molecules [35,36]. However, equilibration of PmB between two states, both capable of interacting with LPS with almost equal affinity, and the transition between them cannot altogether be ruled out at the moment [36,37]. An examination of the structure of PmB reveals

Figure 4 A plot of ΔH_b versus $T\Delta S_b$ at room temperature ($24.9 \pm 0.1^\circ \text{C}$) for PmB–LPS and PmB–lipid A interactions

The values plotted are for *E. coli* 055: B5 LPS (■), *E. coli* 011: B4 LPS (△), *E. coli*: B4 LPS in liposomes (◇), lipid A (▲) from *E. coli* K-12 D31m4 strain, *S. minnesota* Re 595 LPS (□), lipid A in liposomes (○), *S. typhimurium* LPS (●), and *S. enteritidis* LPS (◆). The straight line represents the best least-squares fit of the data to a straight line. Data for the monophosphoryl lipid A from *E. coli* K-12 and the diphosphoryl lipid A from *S. minnesota* Re 595 are not plotted due to the unavailability of values at 24.9°C .

that it has five positive charges, hence one expects a significant contribution to the binding energy from ionic interactions. Several pieces of evidence rule out any significant role for ionic forces in the recognition of LPS by PmB. (i) For ionic interactions, heat capacity changes should have been positive instead of being negative as observed experimentally [38]. As shown in Table 2, increasing salt concentrations should have decreased the ΔG_b° value. At 1.0 M NaCl all ionic interactions can be considered neutralized yet the value of ΔG_b° at this salt concentration is almost the same as without salt [39]. In this respect PmB–LPS interaction differs from the binding of heparin to thrombin [40]. (iii) Equally consistent with the above observation is the finding that ΔG_b° at pH 9.9 is unchanged from its value at pH 7.5 where the NH_2 group of DAB (α, γ -diaminobutyric acid) should be practically unprotonated ($\Delta G_b^\circ = -34 \text{ kJ} \cdot \text{mol}^{-1}$ and $\Delta H_b = 39 \text{ kJ} \cdot \text{mol}^{-1}$ at 24°C for *E. coli* 055: B5 LPS). (iv) Reductively methylated PmB (3.2–3.9 groups/molecule), despite retention of its positive charges, is unable to bind LPS at all. (v) Acetylated and formylated PmB, containing as many as four acyl groups, bound to their targets slightly better than the native PmB molecule (Table 3). Non-essentiality of most of the positive charges of PmB as alluded to above is also consistent with the model of Feingold et al. which proposes requirement of a single charged DAB side-chain for the lytic activity of PmB [41]. Rustici et al. [22] have, likewise, observed that all of the NH_2 groups of PmB complexed with LPS are accessible to picryl sulphate. Taken together these results argue against the involvement of ionic forces in the stabilization of the PmB–LPS complex and instead lead us to propose that a hydrophobic/van der Waals packing component derived from the aliphatic portion of the DAB side-chain might contribute significantly to the thermodynamic properties of PmB–LPS interactions. The positive charge of the NH_2 groups perhaps prevents the lumping together of non-polar amino acid side-chains by electrostatic repulsion, in addition to providing the necessary polarity during the initial stages of recognition. Precedents for the trivial contribution of ionic interaction between charged molecules are available in the literature in the binding of heparin to anti-thrombin and to the basic fibroblast growth factor [39]. Moreover the stoichiometry for the binding of LPS/lipid A with PmB,

Table 2 Effect of salt concentration on the energetics of PmB–lipid A and PmB–LPS associations at 24 °C

Values from average of two experiments.

LPS or Lipid A	NaCl (M)	<i>n</i>	K_b (10^6 M ⁻¹)	ΔH_b (kJ·mol ⁻¹)	$-\Delta G_b^\circ$ (kJ·mol ⁻¹)	ΔS_b (J·mol ⁻¹ ·K ⁻¹)
<i>S. enteritidis</i> LPS	—	0.97	5.22	63	38	341
	0.1	0.99	5.40	63	38	342
	0.3	1.04	6.03	52	39	303
	0.5	1.02	6.12	51	39	302
	0.75	1.00	5.13	50	38	296
	1.0	1.05	5.41	66	38	351
Lipid A	—	1.04	10.22	39	40	265
	0.1	1.01	10.08	40	40	268
	0.3	0.97	10.53	37	40	260
	0.5	0.98	10.35	37	40	258
	0.75	1.05	9.72	40	40	269
	1.0	0.93	9.82	41	40	273

Table 3 Thermodynamic parameters for LPS–acylated PmB derivatives at 24 °CValues are averages from two experiments. Values of *n* were always close to 1, except for PmB derivatives which contained more than four of their amino groups modified.

LPS	PmB	K_b (10^6 M ⁻¹)	ΔH_b (kJ·mol ⁻¹)	$-\Delta G_b^\circ$ (kJ·mol ⁻¹)	ΔS_b (J·mol ⁻¹ ·K ⁻¹)
<i>S. enteritidis</i>	Native	5.22	64	38	344
	1.8 Acetyl	6.84	66	39	353
	2.7 Acetyl	7.53	68	39	360
	3.6 Acetyl	5.70	72	38	371
	4.4 Acetyl	1.51	45	35	270
	1.6 Formyl	6.12	67	38	356
	2.8 Formyl	7.20	72	39	374
	3.3 Formyl	8.11	74	39	383
	4.5 Formyl	1.42	51	35	135

which contains five positive charges, should have been highly variable if it was primarily driven by electrostatic interactions, as LPS and lipid A used here contain variable numbers of anionic groups. For example they contain two (monophosphoryl lipid A from *E. coli*), or four (diphosphoryl lipid A from RE 595 strain of *S. minnesota*) groups, while the lipid A from the deep rough mutant *E. coli* K-12 D31m4 strain and *S. typhimurium* may contain up to 12–14 negative charges [8,42]. Lipid A from *S. typhimurium* also contains pyrophosphoethanolamine and arabinosamine groups [42]. To substantiate our interpretation, we have determined the binding of PmB to a charged amphiphile, dodecanoic acid, where it was observed that each molecule of PmB interacts with five molecules of this negatively charged lipid (Figure 5) ($n = 0.204$; $K_b = 1.8 \times 10^6$ M⁻¹ and $\Delta H_b = -59$ kJ·mol⁻¹). Normalization of these parameters per fatty acid molecule complexed with PmB yields $K_b = 0.36 \times 10^6$ mol⁻¹, $\Delta H_b = -59$ kJ·mol⁻¹ and $\Delta S_b = -146$ J·mol⁻¹·K⁻¹ at 24.6 °C. Unlike the interaction between PmB and LPS, the association of PmB with dodecanoic acid is strongly compromised by salt with ΔH_b and ΔG_b° diminishing till 0.7 M NaCl. More importantly, the ΔC_p for the interaction of PmB with dodecanoic acid is positive ($\Delta C_p = 879$ J·mol⁻¹·K⁻¹ from $\Delta H_b = -17$ kJ·mol⁻¹ at 14.1 °C; values computed after normalization). If electrostatic interactions were the primary force for PmB–endotoxin complex formation then about 2.5 molecules of monophosphoryl lipid A and 1.25 molecules of diphosphoryl lipid A (from the deep rough *E. coli* K-12 strain, D31m4) should have bound to each molecule of PmB. These results thus confirm that the PmB–dodecanoic

acid interaction, in contrast to LPS–PmB associations, is driven by ionic interaction.

This is further substantiated by studies of the binding of PmB to phosphatidic acid, a bi-anionic phospholipid. As expected each PmB molecule complexed with at least two phosphatidic acid molecules. The binding of PmB to phosphatidic acid was almost completely prevented by 1.0 M NaCl and was pH-dependent. Moreover, PmB–phosphatidic acid association displays positive changes in ΔC_p , attesting to the ionic nature of their interaction (G. Jyoti., M. K. Jain and A. Surolia, unpublished work).

It is in order to mention here that the thermodynamic parameters for the binding of PmB with dodecanoic acid both above and below the critical micelle concentration are identical, indicating again that the mode of the presentation of the target molecules does not influence the interaction with PmB. Likewise the thermodynamic parameters for the binding of PmB to lipid A and LPS (3 %) incorporated in dipalmitoylphosphatidylcholine liposomes, where they are expected to be dispersed relatively more uniformly, are identical to those reported for LPS or lipid A in solution (Table 1). These results lend further support to our conclusion regarding the primacy of hydrophobic forces in the recognition of LPS by PmB, and that the mode of their presentation does not appear to alter their interactions with this antibiotic.

The following features of PmB would help explain the stoichiometries and specificities of its interactions with LPS and lipid A molecules and the negatively charged lipids. A con-

Figure 5 Calorimetric titration curve for the binding of PmB to dodecanoic acid

Heat exchange upon titration of PmB (4 mM) into dodecanoic acid (1 mM) in 50 mM phosphate buffer (pH 7.5) at 24.6 °C. (A) Sixteen 9 μ l injections of 6 s duration were made at intervals of 3 min. (B) Integrated areas for the above peaks per mole of the titrant are plotted against the injection number. At the end of experiments the turbidity was barely visible.

sideration of these results together with the available information on the structure of PmB allows us to hypothesize about the features of this molecule which are necessary for its interaction with LPS or lipid A as well as with the negatively charged lipids [43–45]. Since the non-polar region of the molecule interacts with LPS, its stoichiometry is close to one and is independent of pH and salt concentration. The space-filling model of PmB shows that almost all of the amino groups of DAB side-chains are oriented on one side of the molecule while the side-chains of non-polar residues project away on the opposite surface of the molecule (S. Balasubramanian and A. Surolia, unpublished work). Perhaps the methyl groups of threonine residues are also projected on this side of the molecule, imparting additional hydrophobicity to this surface of the molecule. The acyl moiety attached at the N-terminus despite its flexibility perhaps also lies towards the non-polar face of the molecule in its complex with LPS. This is also consistent with the differences in the properties of the PmB nonapeptide and PmB [46,47]. Thus, LPS–PmB interactions are primarily mediated through their hydrophobic surfaces and the positive charges of the DAB side-chains perhaps help in appropriate positioning of the antibiotic molecule at the surface of LPS during the initial stages of the interaction. Stoichiometric binding of colistin to LPS in which D-Leu replaces D-Phe at the sixth position of PmB suggests that it also provides a hydrophobic surface akin to that provided by PmB. On the other hand for the interaction with anionic fatty acids and phospholipids the charged face of PmB interacts with the negatively charged head groups of phospholipids and hence the stoichiometries are equivalent to the number of charged groups on the PmB molecules. This interaction, as expected, is sensitive

to salt concentration in the medium. Moreover, the stoichiometries, K_b and ΔH_b , reduce proportionately for binding to dodecanoic acid upon incorporation of one to three acetyl groups, while PmB with 4.2–4.6 acetyl groups was inactive. These results thus attest to the indispensability of electrostatic interaction in the binding of PmB with negatively charged lipids, in contrast to PmB–endotoxin interaction. Based on these results, the subtle structural features that allow the binding of PmB to LPS include a cluster of non-polar amino acid side-chains exposed to the solvent on one side of the molecule while the positively charged residues are oriented away from them on the other side of the peptide. Though positive charges *per se* are not exclusively mediating the interaction, they are perhaps involved in optimally orienting the non-polar components of these charged residues. (Inactivity of reductively methylated PmB appears to be due to the perturbation of the segregated distribution of the non-polar and polar surfaces of the molecule.) The exact features regarding the orientation of positive charges in PmB and colistin are not obvious at the moment. However, there are several other proteins and peptide structures which display similar surface characteristics to those proposed here for PmB. Colicin A has a cluster of basic residues located on one surface of the molecule which help orient it at the membrane while the other face of the molecule, which is distinctly non-polar, is crucial for its pore-forming activity [48]. Defensins also exhibit exposed cationic and non-polar residues oriented at the different faces of the molecule. The crystal structure of human defensin HNP-3, for example, reveals that it is a basket-shaped molecule which is distinctly amphiphilic in nature. It has hydrophilic residues confined to the ‘top’ of the basket, while the hydrophobic residues are at the bottom [49]. The model proposed here for the exposed non-polar and cationic surfaces, which are displayed at the disparate regions of the PmB molecule, account fully for the physico-chemical properties of this antibiotic. These include its binding and fusion of negatively charged liposomes, haemolytic activity, etc. [41,50]. The observation that even pentyllysine, containing six positive charges as opposed to the five of PmB, despite its competence to aggregate negatively charged liposomes fails to fuse them [51] strengthens further our hypothesis regarding the mode of action of PmB. More importantly, a consideration of the structural features reported for *Limulus* anti-LPS factor (LALF) [52] prompts us to suggest that a ring of positively charged and exposed hydrophobic residues on distinct surfaces constitute a general motif for binding of molecules to LPS. We believe that the optimization of the asymmetric distribution of the non-polar and the positively charged amino acid side-chains on the opposite faces of the peptides would lead to improved energetics and design of such molecules for combating endotoxicosis.

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