Temperature Dependence of the Binding of Endotoxins to the Polycationic Peptides Polymyxin B and Its Nonapeptide

Klaus Brandenburg,* Alexander David,* Jörg Howe,* Michel H. J. Koch,† Jörg Andrä,* and Patrick Garidel‡
*Forschungszentrum Borstel, Leibniz-Zentrum für Medizin und Biowissenschaften, D-23845 Borstel, Germany; †European Molecular Biology Laboratory, Deutsches Elektronen-Synchrotron, D-22603 Hamburg, Germany; and ‡Martin-Luther-Universität Halle/Wittenberg, Institut für Physikalische Chemie, D-06108 Halle/Saale, Germany

ABSTRACT The interaction between endotoxins—free lipid A and various lipopolysaccharide (LPS) chemotypes with different sugar chain lengths—and the polycationic peptides polymyxin B and polymyxin nonapeptide has been investigated by isothermal titration calorimetry between 20 and 50°C. The results show a strong dependence of the titration curves on the phase state of the endotoxins. In the gel phase (<30°C for LPS and <45°C for lipid A), an endothermic reaction is observed, for which the driving force is an entropically driven endotoxin-polymyxin interaction, due to disruption of the ordered water structure and cation assembly in the lipid A backbone and adjacent molecules. In the liquid crystalline phase (>35°C for LPS and >47°C for lipid A) an exothermic reaction takes place, which is mainly due to the strong electrostatic interaction of the polymyxins with the negative charges of the endotoxins, i.e., the entropic change ΔS is much lower than in the gel phase. For endotoxins with short sugar chains (lipid A, LPS Re, LPS Rc) the stoichiometry of the polymyxin binding corresponds to pure charge neutralization; for the compounds with longer sugar chains (LPS Ra, LPS S-form) this is no longer valid. This can be related to the lower susceptibility of the corresponding bacterial strains to antibiotics.

INTRODUCTION

Lipopolysaccharides (LPS) are the main amphiphilic components of the outer leaflet of the outer membrane of Gram-negative bacteria. Due to their ability to induce a variety of biological effects in mammals, in particular the production of proinflammatory cytokines, they are called endotoxins (Mamat et al., 1999). This term, however, may be somewhat misleading because at low endotoxin concentrations, the biological effects may be beneficial, because cytokines such as tumor-necrosis-factor-α (TNFα) have been shown to possess antitumor activity. Only at higher endotoxin concentrations does self-poisoning of the body occur due to an overflowing production of cytokines eventually resulting in the septic shock syndrome and multiorgan failure (Alexander and Rietschel, 2001).

Depending on the type of bacterial mutant, rough mutants or smooth forms, LPS consists of a sugar portion with varying length of oligo- or polysaccharide chains. The sugar moiety is covalently linked to the hydrophobic moiety of LPS, lipid A, which anchors the LPS molecule to the membrane. For enterobacterial LPS, lipid A is composed of a diglucosamine backbone that is phosphorylated in positions 1 and 4′ and acylated by six to seven hydrocarbon chains ester- and amide-linked in positions 2, 3 and 2′, 3′ (Zähringer et al., 1999). For other bacterial genera, the lipid A structure of LPS may differ in particular with respect to the acylation pattern. For all enterobacterial as well as most nonenterobacterial strains, two very unusual sugars 2-keto-3-deoxyoctonate, each having one carboxylate group, are linked to the lipid A moiety. Thus, most LPS backbones carry a large number of negative charges.

On the basis of these structural facts, polycationic drugs (peptides, proteins, and/or antibiotics) such as polymyxin B (PMB), a polycationic decapeptide, may be assumed to effectively bind to LPS and lipid A and thus should be able to protect against the pathophysiological effects of LPS (Schindler and Osborn, 1979; Hancock, 1997; Scott and Hancock, 2000). However, a systematic application of PMB to combat sepsis is severely restricted by virtue of its toxicity at higher concentrations.

A variety of studies describe the effects of PMB on outer membranes of intact bacteria as well as on isolated LPS or anionic phospholipids (Kubesch et al., 1987; Schröder et al., 1992; Vaara, 1992; Wiese et al., 1998), which give insights in the underlying peptide-endotoxin interactions.

The detailed study of the interaction mechanisms of drugs with target structures such as LPS requires the application of a variety of physical techniques. One of them, isothermal titration calorimetry (ITC), allows the determination of thermodynamic parameters associated with the binding process as well as the binding stoichiometry. In the frame of biophysical studies on the interaction of PMB and its nonapeptide derivative PMBN with LPS from various strains, titration curves corresponding to exothermic reaction were observed (Brandenburg et al., 2002a,b). As a model of LPS-PMB interaction, a two-step mechanism was proposed consisting of electrostatic binding of the positive charges of PMB to the negative charges of LPS followed by a hydrophobic interaction of the small acyl chain of PMB

Submitted June 20, 2004, and accepted for publication November 30, 2004.

Address reprint requests to Dr. Klaus Brandenburg, Forschungszentrum Borstel, LG Biophysik Parkallee 10, D-23845 Borstel, Germany. Tel.: 49-04537-188235; Fax: 49-04537-188632; E-mail: kbranden@fz-borstel.de.

0006-3495/05/03/1845/14 $2.00

doi: 10.1529/biophysj.104.047944

with the hydrocarbon moiety of LPS. However, Surolia and co-workers (Srimal et al., 1996; Thomas and Surolia, 1999) describe the LPS-PMB interaction as noncooperative and endothermic, predominantly hydrophobic in nature and entropy driven, and with negligible contributions from electrostatic interactions. The main difference between our measurements and those of the Surolia group lies in the measuring temperature, 37°C for the former and 20°C for the latter. Temperature may, however, be extremely important with respect to the phase state of LPS, because all enterobacterial LPS have a gel to liquid crystalline phase transition in the temperature range 30–37°C (Brandenburg and Seydel, 1990). Thus, the measurements of the Surolia group were made in the gel state (β) of the hydrocarbon chains, whereas our investigations were performed under near physiological conditions with the LPS being in the liquid crystalline (α) phase of the acyl chains. In this article, besides resolving these contradictions we have performed a systematic ITC study of the temperature dependence of the endotoxin-PMB binding. The phase behavior of various endotoxin-peptide mixtures was investigated systematically by differential scanning calorimetry (DSC). Polymyxin nonapeptide (PMBN) as well as PMB systems were investigated to study the influence of the small acyl chain present in PMB but absent in PMBN toward the phase behavior of the endotoxin. To study the influence of the polar headgroup, which is formed by sugar moieties, we have used a variety of endotoxin structures including lipid A and rough and smooth form LPS from *Salmonella minnesota*. The phospholipid dioleoylphosphatidylcholine (DOPC) was also tested as reference. In concomitant Fourier-transform infrared spectroscopic (FTIR) measurements, the gel to liquid crystalline phase behavior of the respective compounds was monitored, and the aggregate structure of a selected LPS compound was also determined by small-angle x-ray diffraction using synchrotron radiation.

The results show that the binding of PMB, but also PMBN, to endotoxins is phase specific leading to a conversion of the exothermic reaction observed in the liquid crystalline phase into an endothermic one in the gel phase. Additionally, an influence of the sugar structures on the endotoxin-peptide affinity was also observed. The later effect has a large impact on the bactericidal activity of the peptide interaction.

MATERIALS AND METHODS

LPS and lipids

Lipopolysaccharides from various rough mutant strains Re to Ra from *S. minnesota* were extracted from bacteria grown at 37°C by the phenol/chloroform/petrol ether method (Galanos et al., 1969) and smooth-form LPS from *Salmonella abortus equi* by the phenol/water technique (Westphal et al., 1952). LPS samples were purified and lyophilized. Their chemical structures are given in Fig. 1 *A*. The LPS were used usually in their natural salt form, for LPS Re; also the Mg^{2+} salt form was used, obtained by extensive dialysis for 48 h against 100 mM $MgCl_2$.

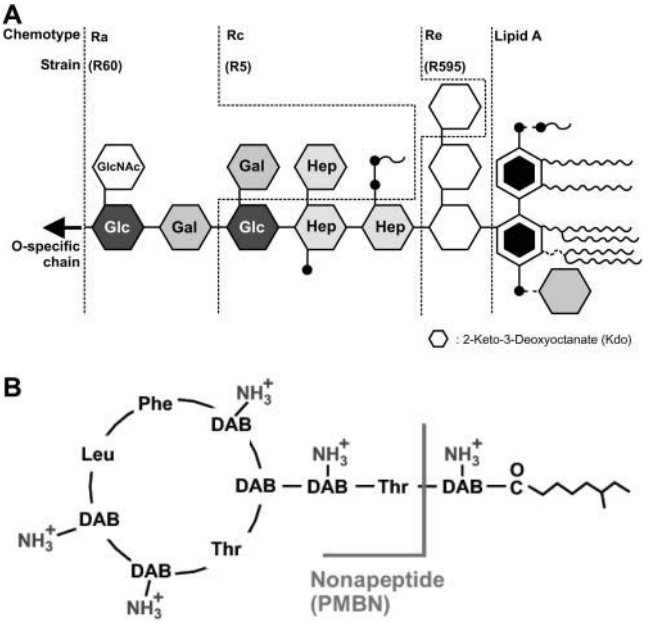

FIGURE 1 Chemical structures of (*a*) various rough mutant and S-form LPS from *S. minnesota*. Additional phosphates attached to the heptoses are present only for LPS Ra and S-form LPS. Abbreviations are: Hep, L-*glycero*-D-*manno*-heptopyranose; Glc, glucose; Gal, galactose. (*b*) Polymyxin B and its nonapeptide PMBN. DAB, diamino butyric acid.

Free lipid A was isolated from LPS Re by acetate buffer treatment (Seydel et al., 2000). After isolation, the resulting lipid A was purified and converted into the triethylamine salt form.

The known chemical structure of lipid A from LPS Re was checked by the analysis of the amount of glucosamine, total and organic phosphate, and the distribution of the fatty acid residues applying standard procedures as well as modern mass spectrometric analysis (kindly performed by B. Lindner, Forschungszentrum Borstel, Biophysics Division). The amount of 2-keto-3-deoxyoctonate was also checked and in no case exceeded 5%.

The chemical structures of the investigated LPS are given in Fig. 1 *A*. DOPC was purchased from Avanti Polar Lipids (Alabaster, AL); PMB and its nonapeptide PMBN (chemical structures shown in Fig. 1 *B*) were purchased from Sigma (Deisenhofen, Germany).

Sample preparation

The lipid samples were usually prepared as aqueous dispersions at high buffer (20 mM Hepes, pH 7.0) content, depending on the sensitivity of the technique: 0.05 or 0.15 mM for the ITC experiments, 0.4 mM for the DSC, and 40 mM for the x-ray experiment. In some cases the lipids were also prepared in physiological saline (150 mM NaCl in 20 mM Hepes). In all cases, the lipids were suspended directly in buffer, sonicated, and temperature-cycled several times between 5 and 70°C and then stored at least 12 h at 4°C before measurement.

Isothermal titration calorimetry

Microcalorimetric experiments of peptide binding to endotoxins were performed on a MCS isothermal titration calorimeter (Microcal, Northampton, MA) at 37°C. The endotoxin samples at a concentration of 0.05–0.15 mM—prepared as described above—were filled into the microcalorimetric cell (volume, 1.3 ml) and the peptides in the concentration range 0.5–5 mM into the syringe compartment (volume, 100 μl), each after thorough

degassing of the suspensions. After thermal equilibration, aliquots of 3 μl of peptide solution were added every 5 min into the lipid-containing cell, which was stirred constantly, and the heat of interaction after each injection measured by the ITC instrument was plotted versus time. The total heat signal from each experiment was determined as the area under the individual peaks and plotted versus the [peptide]/[lipid] molar ratio. Because the instrument works in temperature equilibrium at a constant ''current feedback'' corresponding to a power of ~74 μW, the occurrence of an exothermic reaction leads to a lowering of this current and of an endothermic reaction to an increase. All titration measurements, performed at constant temperatures, were repeated at least four times.

As control for all ITC experiments, PMB and PMBN were titrated into pure buffer; however, only a negligible enthalpic reaction due to dilution could be observed (data not shown).

Differential scanning calorimetry

Differential scanning calorimetry measurements were performed with a MicroCal VP scanning calorimeter (MicroCal). The heating and cooling rates were 1°C/min. Heating and cooling curves were measured in the temperature interval from 10 to 100°C. The phase transition enthalpy is obtained by integration of the heat capacity curve as described previously (Jürgens et al., 2002). Usually, three consecutive heating and cooling scans were measured. The lipid dispersion was prepared according to recently described protocols at a concentration of ~1 mg/ml (corresponds to 0.5 mM) (Blume and Garidel, 1999).

X-ray diffraction

X-ray diffraction measurements were performed at the European Molecular Biology Laboratory outstation at the Hamburg synchrotron radiation facility HASYLAB using the double-focusing monochromator-mirror camera X33 (Koch and Bordas, 1983). Diffraction patterns in the range of the scattering vector $0.07 < s < 1$ nm^{-1} ($s = 2 \sin \theta / \lambda$; 2θ scattering angle and λ, the wavelength, = 0.15 nm) were recorded at various temperatures with exposure times of 2 or 3 min using a linear detector with delay line readout (Gabriel, 1977). The wavelength calibration was done with tripalmitate as a standard having a periodicity of 4.06 nm at room temperature. Further details of the data acquisition and evaluation system is described elsewhere (Boulin et al., 1986). In the diffraction patterns presented here, the logarithm of the scattering intensity log$I$ is plotted versus the scattering vector $s = 1/d$ (d = spacing). The x-ray patterns were evaluated as described previously (Brandenburg et al., 1998) assigning the spacing ratios of the main scattering maxima to defined three-dimensional structures.

RESULTS

Isothermal titration of the system LPS Re: PMB and PMBN

In a former ITC investigation on LPS-PMB interactions, the experiments were performed exclusively at the physiologically relevant temperature of 37°C. In this study, the temperature range of ITC measurements was extended from 20 to 40°C. The microcalorimetric titration of a 0.05 mM LPS Re (from *S. minnesota* R595) dispersion with 1.5 mM PMB at 20°C is shown in Fig. 2. At the beginning of the titration experiment (injection of 3 μl every 5 min) there is a strong endothermic reaction that decreases after the 12th to 13th injection. The absolute amount of LPS Re in the cell is 65×10^{-6} mol, and after the 13th injection the absolute amount of peptide in the cell is 54×10^{-6} mol. Due to the fact that the peptide bears five positive and LPS four negative charges, the decrease in the heat response to very low values indicates saturation of the reaction. This value apparently corresponds to charge compensation, because four PMB molecules with a total of 20 positive charges would bind to five LPS Re molecules with a total of 20 negative charges. At the end of the titration experiment, the small heat contribution measured corresponds to the negligible heat of dilution. In Fig. 3 *A* (*top left*), the enthalpy change ΔH_c is plotted versus the [PMB]/[LPS Re] ratio showing saturation values around 0.8. At 20°C a gel phase peptide interaction is measured (see also Fig. 7 *A*). From this binding behavior it can be assumed that the electrostatically driven binding process is superimposed by other effects, because a purely electrostatic attraction should lead to an exothermic reaction (Carneiro et al., 2002) as has also been observed for the interaction of divalent cations with negatively charged liposomes in the gel as well as in the liquid crystalline phase (Garidel and Blume, 1999). Thus, a more complicated mechanism of action must accompany binding.

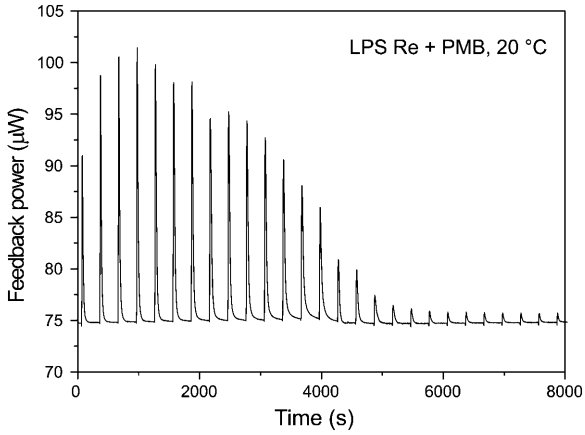

FIGURE 2 Isothermal calorimetric titration of LPS Re (0.15 mM) from *S. minnesota* strain R595 with PMB (3 mM) at 20°C. For this, the LPS dispersion in the calorimetric cell was titrated every 5 min with 3 μl of PMB. The increase in the feedback power indicate an endothermic reaction.

The temperature dependence of the ITC titration in the range 20–40°C is shown in Fig. 3 *A*. The endothermic reaction is observed up to 28°C, decreases significantly at 31°C, and converts into an exothermic reaction at 36–40°C. Interestingly, the binding stoichiometry remains temperature independent around [PMB]/[LPS Re] = 0.8 molar ratio. The behavior of the conversion from an endothermic into an exothermic reaction seems to be related to the phase behavior of LPS Re with an acyl chain phase transition temperature T_c lying around 31°C (Brandenburg and Seydel, 1990). Above T_c, the exothermic binding reaction of the positive charges of the peptide to the negative charges of LPS Re seems to be dominant.

To determine the influence of the acyl chain of PMB, similar experiments were performed with the nonapeptide

PMBN. The results (Fig. 3 *B*) yield also a phase specificity, an endotherm below and an exotherm above T_c. However, the enthalpy changes at $T < T_c$ (25°C) are much lower than found for the LPS Re-PMB endotherm. It should be mentioned that the registered interaction enthalpy ($T >$ 25°C) also contains contributions due to an isothermally induced phase transition into the liquid crystalline phase due to peptide binding. As can be seen from Fig. 7 *B*, the maximum of the phase transition temperature of LPS Re is dramatically shifted to lower temperature in the presence of PMBN, indicating a destabilization of the gel phase.

So far, experiments were performed in buffer (20 mM Hepes). To test the physiological validity of our findings, titration experiments were also performed in physiological saline (150 mM NaCl in 20 mM Hepes). A comparison of the results obtained at 24°C for the [PMB]/[LPS Re] system in pure buffer and in saline is shown in Fig. 4. Both curves exhibit similar characteristics, only the maximum enthalpy changes are lower and a slightly higher [PMB]/[LPS] ratio is needed for saturation of binding in the presence of the saline. Parallel to the other diagrams shown in Fig. 3 *A*, the comparison of the PMB-LPS interaction at temperatures $>T_c$

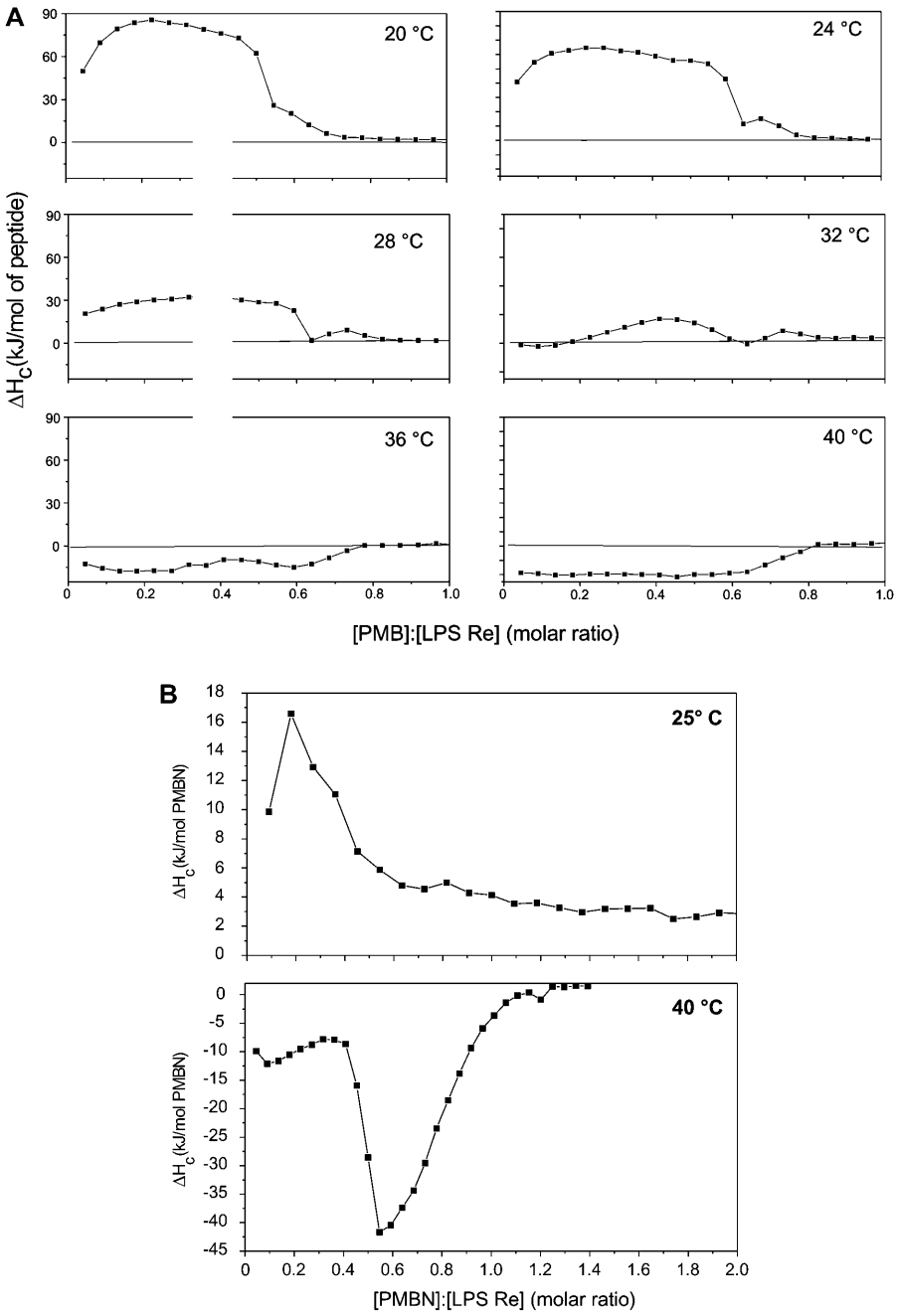

FIGURE 3 Enthalpy change of the (*a*) LPS Re-PMB reaction versus [PMB]/[LPS Re] molar ratio in the temperature range 20–40°C, and (*b*) of the LPS Re-PMBN reaction versus [PMBN]/[LPS Re] molar ratio at temperatures 25 and 40°C.

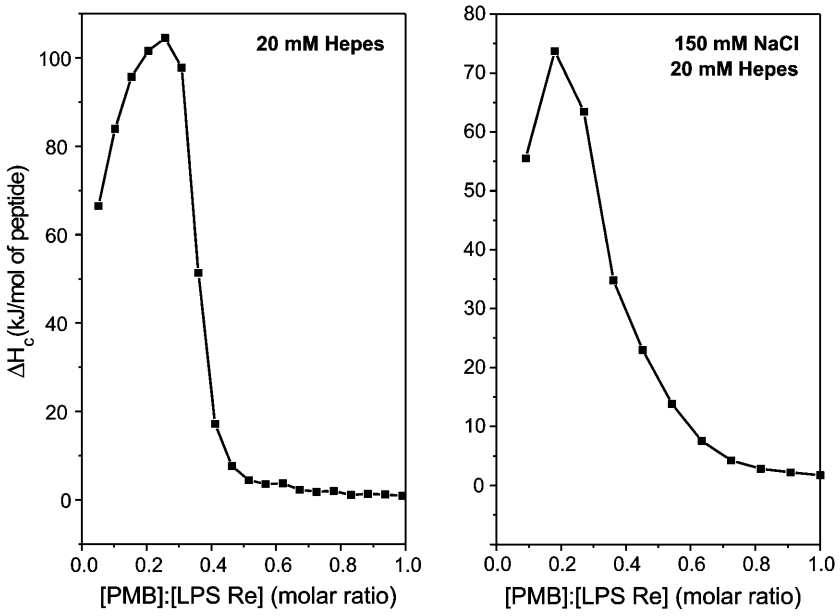

FIGURE 4 Enthalpy change of the LPS Re-PMB reaction versus the [PMB]/[LPS Re] molar ratio at 24°C, in pure Hepes buffer (*left*) and in physiological saline (*right*).

for buffer and saline shows in accordance with Fig. 3 *A* exothermic enthalpy changes with a similar overall course (data not shown).

Calculation of thermodynamic parameters

The ITC binding curves were analyzed applying common laws of thermodynamics for the calculation of $T\Delta S$ and the Gibbs free energy ΔG (Jelesarov and Bosshard, 1999). The results are shown in Fig. 5, and prove a strong decrease of $T\cdot\Delta S$ and, thus, of the entropy, despite increasing T. Interestingly, the free energy ΔG exhibits only small changes.

The temperature dependence of the binding enthalpy usually is assumed to allow the determination of the change in heat capacity Δc_p using the Kirchoff equation: $\Delta c_p = d(\Delta H_c)/d(T)$ (Loladze et al., 2001; Garidel and Blume, 1999; Gallagher and Sharp, 1998). This parameter should describe hydration/dehydration effects that occur during the interaction. In our case it should be the difference in heat capacities between the LPS Re with its counter ions and hydration shell and the free peptide with its counter ions and hydration shell on one hand and the endotoxin-peptide complex on the other hand. The observed Δc_p values can be interpreted as the sum of changes of heat capacities in the polar and apolar region of the interaction partners (Garidel and Blume, 1999). As can be seen in Fig. 5, with increasing temperature, the binding enthalpy decreases and becomes even negative. Thus, from the temperature dependency of ΔH_c a negative change in heat capacity can be calculated for both phases. In contrast, from the course of ΔG with temperature in Fig. 5 an increase of c_p with temperature can be deduced.

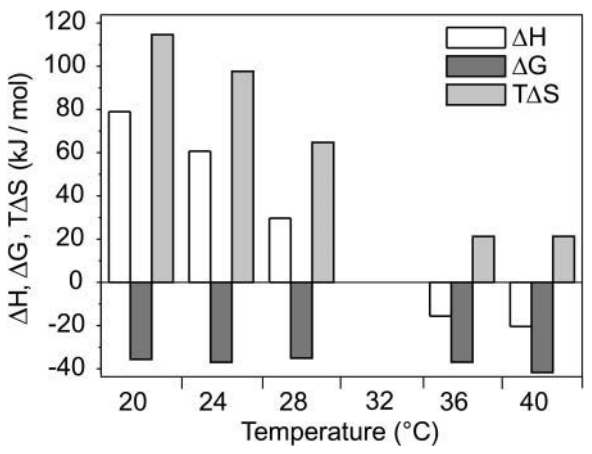

FIGURE 5 Temperature dependence of ΔH, ΔG, and $T\Delta S$ for the [LPS Re]/[PMB] binding. The presented data express only a tendency; a more precise treatment would imply more measuring points within each phase.

It should be noted that in Fig. 5 the temperature dependence of the thermodynamic quantities represents only a tendency; a rigorous treatment including a precise error analysis would require some more measuring points within each phase.

Synchrotron radiation x-ray diffraction of LPS aggregates

Addition of PMB and PMBN converts the aggregate structure of LPS Re from a unilamellar/cubic into a multilamellar one, which might provide an enthalpic contribution to the binding (Brandenburg et al., 2002b). For an assessment of this contribution, ITC measurements were also performed with the Mg^{2+} salt form of LPS Re. This sample adopts only multilamellar structures in the relevant temperature range, in particular below and above $T_c = 31°C$, which

can be deduced by synchrotron radiation x-ray diffraction measurements (Fig. 6). The diffraction patterns are clearly indicative of reflections at equidistant ratios at all temperatures. For example, at 40°C the diffraction peaks corresponding to the first four orders have spacings at 5.94, 2.97, 1.98, and 1.48 nm, respectively. In ITC measurements the binding characteristics of this system (in the presence of Mg^{2+}) is similar to those of the natural salt form of LPS Re, i.e., addition of PMB induces a calorimetric endotherm of ~60 kJ/mol at 25°C and an exotherm of −30 kJ/mol at 50°C (data not shown). Again, negative Δc_p's are obtained for both phases. Thus, it is assumed that reaggregation effects do not seem to contribute to an overall enthalpic effect.

DSC data of the $\beta \leftrightarrow \alpha$ acyl chain melting of LPS Re and lipid A

For a more detailed analysis of the $\beta \leftrightarrow a$ acyl chain melting transition, DSC experiments were performed with LPS Re and the two peptides PMB and PMBN at various molar ratios (Fig. 7). Clearly, with increasing peptide concentrations the calorimetric endotherm of the LPS Re gel to liquid crystalline phase transition becomes broader and shifts to lower temperatures, indicating a destabilization of the gel phase. Additionally, a shoulder at the low-temperature side of the heat capacity is observed for low endotoxin/peptide ratios, indicating a demixing of the phase, and can be interpreted as the formation of endotoxin-peptide clusters. With increasing amount of peptide, the phase transition enthalpy decreases. At a molar ratio of [LPS]:[PMB] = 1:0.8, which corresponds to charge neutralization (four negative charges from LPS and five positive from PMB) (Table 1), the endotherm nearly vanishes (Fig. 7 *A*). This effect is more pronounced in the presence of PMB compared

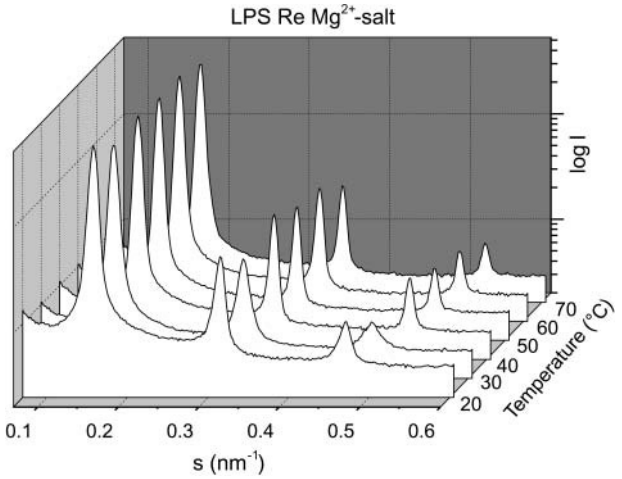

FIGURE 6 Synchrotron radiation small-angle x-ray diffraction patterns of the Mg^{2+}- salt form of LPS Re at 90% water content in the temperature range 20–70°C. The logarithm of the scattering intensity log $I(s)$ is plotted versus the scattering vector $s = 1/d$ (d-spacings in nm).

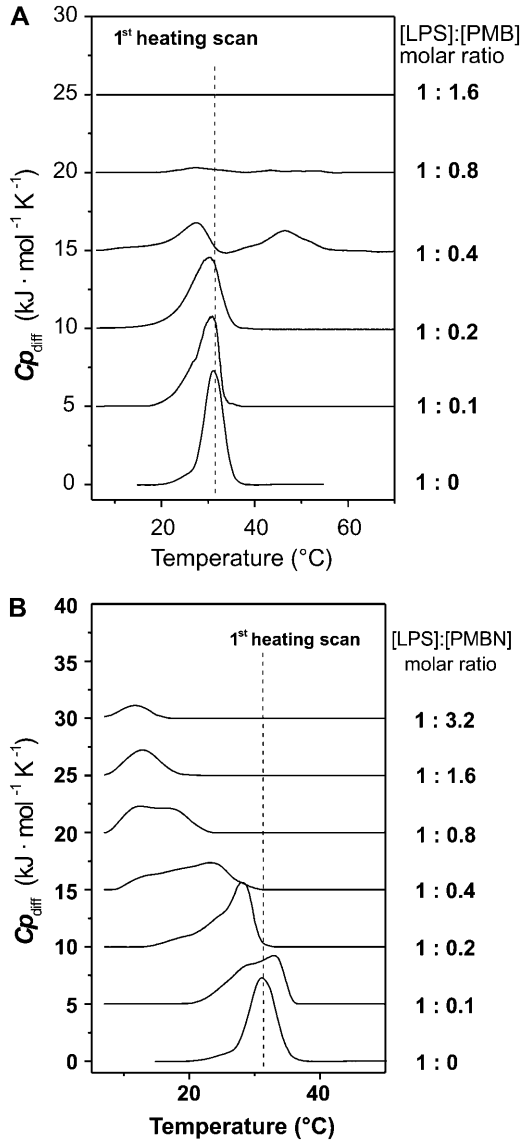

FIGURE 7 Differential scanning calorimetric heat capacity curves of mixtures of LPS and PMB (*a*) and LPS and PMBN (*b*) in various molar ratios. The specific excess heat capacity $c_{p,diff}$ is plotted versus temperature. LPS concentration was 1 mg/ml (corresponding to 0.25 mM).

to PMBN. The results differ for [LPS Re]/[PMBN]; LPS still exhibits, even at a molar ratio [LPS Re]/[PMBN] 1:3.2, acyl chain melting, but at considerably lower temperatures (Fig. 7 *B*). Analogous experiments were also performed with [lipid A]/[peptide] mixtures. Pure lipid A (two negative charges under physiological conditions) exhibits a sharp endotherm, with a smaller area (enthalpy change), which nearly disappears at a molar ratio [lipid A]/[PMB] 1:0.4 (data not shown), again corresponding to charge compensation (see Table 1). In contrast again, for [lipid A]/[PMBN] the endotherm at 44°C does not disappear even at a molar ratio of 1:0.8, but rather is shifted to lower temperatures (34°C) (data not shown). Thus, it can be summarized, that at charge

TABLE 1 Thermodynamic data of the [LPS Re]/[PMB] system and [Lipid A]/[PMB] obtained from differential scanning calorimetry

[LPS Re]/[PMB] (molar ratio)	[LPS Re]/[PMB] (charge ratio −/+)	T_c / °C	$T_{1/2}$ / °C	ΔH_c / kJ mol^{-1}
1:0	0	31.0	4.8	39
1:0.1	8:1	30.8	5.5	37
1:0.2	4:1	30.2	7.3	36
1:0.4	2:1	27.4 and 46.5	7.1 and 9.0	15 and 12
1:0.8	1:1	~27	~10	4
1:1.6	1:2	No	No	No phase transition
[Lipid A]/[PMB] (molar ratio)	[Lipid A]/[PMB] (charge ratio −/+)	T_c / °C	$T_{1/2}$ / °C	ΔH_c / kJ mol^{-1}
1:0	0	44.0	3.7	43
1:0.1	4:1	43.1	7.1	40
1:0.2	2:1	42.5	6.1	26
1:0.4	1:1	41.3	15.8	5
1:0.8	1:2	29.9	~6	4
1:1.6	1:4	No	No	No phase transition

neutralization the PMB-endotoxin phase behavior is nearly completely abolished. Much higher amounts of PMBN are required to induce the same effect, indicating that the interaction mechanisms of both peptides with the endotoxins is different.

In Table 1 the thermodynamic data, molar, and charge ratios, T_c and transition half-value $T_{1/2}$, and ΔH_c, of the LPS Re- and lipid A-PMB interaction from the DSC experiments are listed.

Isothermal titration of other LPS chemotypes with PMB

In further experiments other endotoxin preparations were also tested. The results for the [PMB]/[lipid A] system, considering that T_c of the latter is observed around 43–45°C (Brandenburg and Seydel, 1990), are presented in Fig. 8 in the temperature range 30–50°C. Again, below T_c at 30 and 40°C, clearly an endothermic reaction is observed whereas above T_c, exothermic reactions are measured. The binding stoichiometry lies around [PMB]/[lipid A] = 0.4:0.5; i.e., this corresponds, considering the two negative charges of lipid A, again to charge neutralization with a slightly higher amount of PMB required for saturation.

For the system [PMB]/[LPS Rc] (from *S. minnesota* R5), the latter having a longer sugar oligosaccharide side chain (see Fig. 1), again a conversion from endotherm reactions into exothermic ones can be deduced from the ITC data (Fig. 9). The binding stoichiometry [PMB]/[LPS Rc] = 1.0–1.1 indicates that slightly more PMB is needed than for a pure charge compensation to obtain saturation of binding.

As the last examples in the series of lipopolysaccharides from *S. minnesota* strains, the results are given for LPS Ra (strain R60) and S-form. These LPS have T_c values lying around 37°C (Brandenburg and Seydel, 1990). The ITC curves are presented in Fig. 10. Interestingly, for LPS Ra only exothermic reactions take place (Fig. 10 *A*), there is no conversion correlated with the phase transition as observed for LPS with shorter sugar chains. To check whether a PMB-induced isothermal phase change can be made responsible for this observation, also DSC scans of the [LPS Ra]/[PMB] system were performed (data not shown). Analogous to the [LPS Re]/[PMB] system (Fig. 7 *A*), however, no phase change was found in particular for the measurement at 20°C, at which LPS Re remains in the gel phase.

Binding saturation occurs at a ratio [PMB]/[LPS Ra] = 1.8 at $T < T_c$, and at 0.8 at $T > T_c$. Considering the six negative charges of LPS Ra, at $T < T_c$ more PMB molecules must be present than necessary for a complete charge compensation, whereas the situation is reversed for $T > T_c$, suggesting that saturation takes place before charge compensation.

For LPS S-form, at 20°C there is also partially an endothermic reaction, for the other temperature only exothermic reactions are observed (Fig. 10 *B*). Below 20°C LPS S-form is in the gel phase and the binding of the peptide induces the formation of an endotoxin-peptide complex in its gel phase. At 30°C the pure LPS S-form is still in its gel phase, however, the formed complex is in its liquid crystalline phase. Thus, an isothermal phase transition due to peptide binding is induced. At higher temperature the binding occurs in the liquid crystalline without inducing an isothermal phase transition.

The binding stoichiometry cannot precisely be defined, because LPS S-form is a complex heterogeneous mixture of different substructures, with the bioactive compound corresponding to a LPS Ra or Rb (Galanos et al., 1988).

From these data it can be concluded that the sugar chain length is an important determinant for the kind of thermal reaction.

Other experiments

The possibility that the aggregate structures of the endotoxin-PMB complexes differ below and above T_c was tested

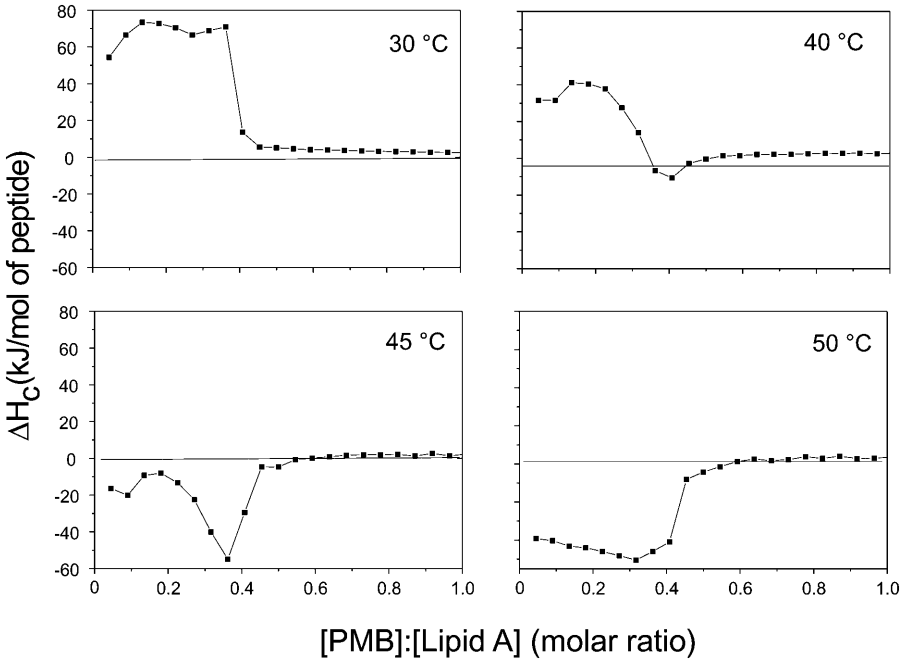

FIGURE 8 Enthalpy change of the lipid A-PMB reaction versus [PMB]/[lipid A] molar ratio in the temperature range 30–50°C. Binding saturation occurs at a molar ratio of 0.4:0.5.

using synchrotron radiation small-angle x-ray diffraction. For lipid A and for short sugar-chain LPS, unilamellar or mixed unilamellar-nonlamellar structures are observed, which convert more and more into multilamellar aggregates with increasing concentrations of PMB. Regarding a possible enthalpic contribution of the reaggregation, former findings indicate this far beyond ($\ll 1\%$) the enthalpic reaction observed here (Brandenburg et al., 2002a,b).

For LPS with longer sugar chains, the data indicate a lower tendency for multilamellar aggregates (data not shown). More importantly, however, the aggregate structures are identical below and above T_c. Therefore, an influence of the change of the aggregate structure due to peptide binding on the measured enthalpy changes can be ruled out.

As control experiment, the interaction of PMB to a non-binding lipid, the uncharged, zwitterionic phospholipid DOPC was studied. It was selected because of the absence of specific interaction between PCs and PMB (Thomas et al., 1999) and the acyl chains are exclusively in the fluid phase between 10 and 50°C (see data bank LIPIDAT). In the ITC

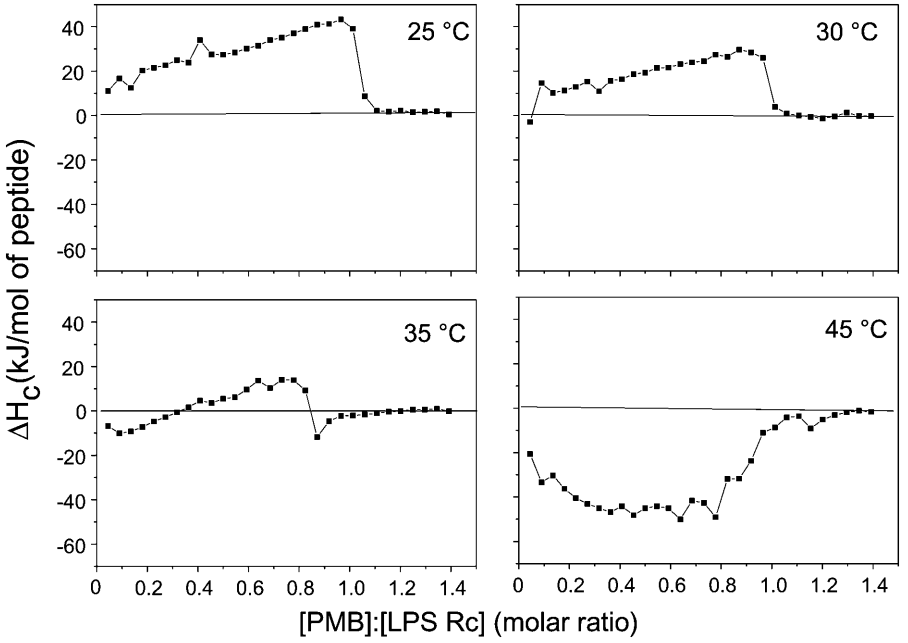

FIGURE 9 Enthalpy change of the LPS Rc-PMB reaction versus [PMB]/[LPS Rc] molar ratio in the temperature range 25–45°C. Binding saturation occurs at a molar ratio of 1.0.

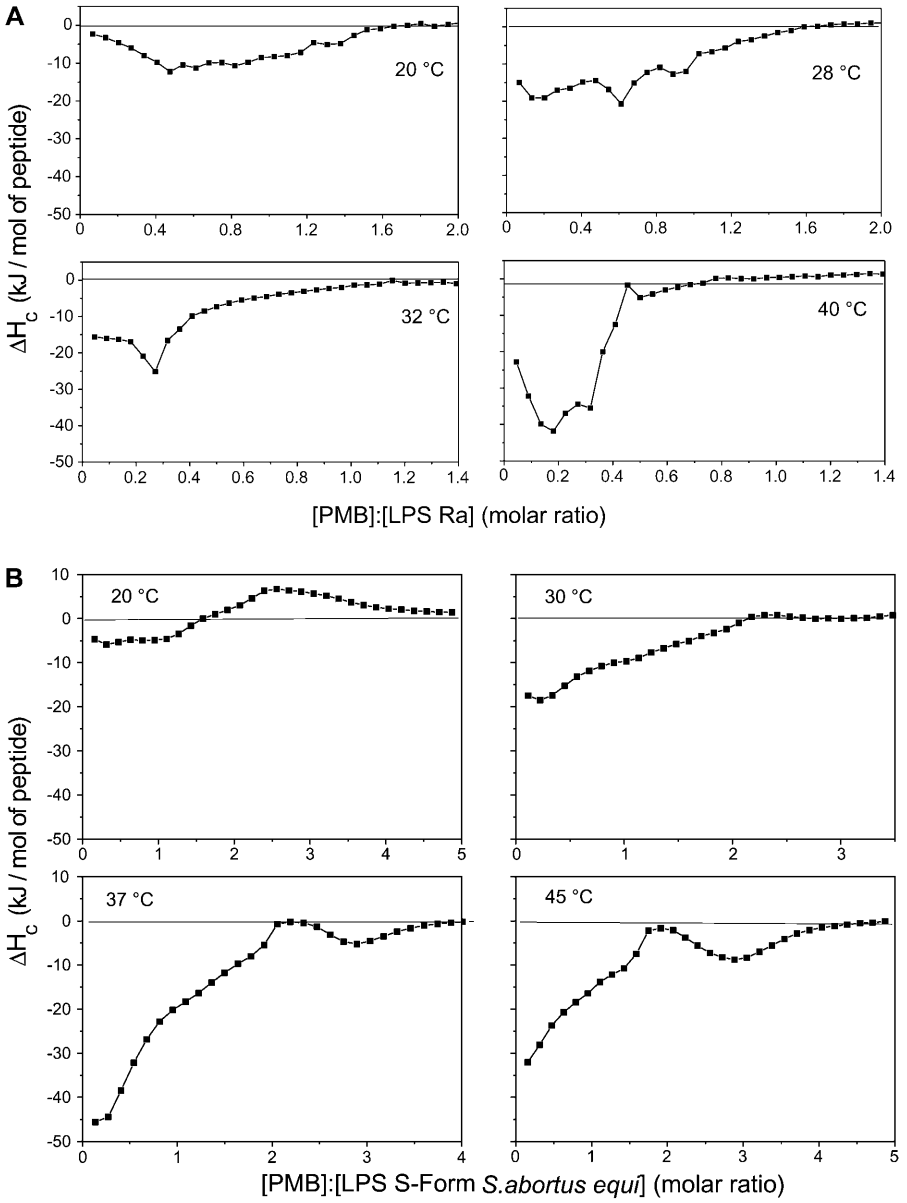

FIGURE 10 (*a*) Enthalpy change of the LPS Ra-PMB reaction versus [PMB]/[LPS Ra] molar ratio in the temperature range 20–40°C. (*b*) Enthalpy change of the LPS S-form-PMB reaction versus [PMB]/[LPS S-form] molar ratio in the temperature range 20–45°C. Binding saturation occurs at a molar ratio of 1.6:1.8 in the gel phase, and around 0.8 in the liquid crystalline phase.

experiments, DOPC shows only an exothermic reaction both at 20 and 45°C (Fig. 11), however, there is clearly a temperature dependence, because the enthalpy changes in the [PMB]/[DOPC] molar range of 0.0–0.4 at 45°C are nearly twice that at 20°C.

DISCUSSION

The main motivation to study the molecular mechanisms of the interaction of polymyxins and other antibacterial peptides with endotoxin aggregates arises from the observation that LPS liberated from bacterial cells presents a serious problem in sepsis research, causing an overflowing production of cytokines (Brade et al., 1999). Thus, an effective antibiotic must kill bacteria as well as neutralize free LPS.

We have studied recently the binding of PMB to lipid A and different rough mutant and smooth form LPS with ITC and complementary techniques, at 37°C. Except for lipid A, for which an endothermic reaction was described, all LPS preparations exhibited clear exothermic reactions (Brandenburg et al., 2002b). These findings are extended by this investigation, showing that the binding effects are strongly phase dependent. We, but also others (Koch et al., 1999; Brandenburg et al., 2002a,b) have interpreted these and similar data as resulting from an electrostatic interaction as the first step of binding between LPS and PMB, corresponding to the attraction of the positive charges of the peptide to the negatively charged lipid A phosphates.

Secondly, a penetration of the small acyl chain of PMB into the LPS membrane takes place. A closer look at the ITC data

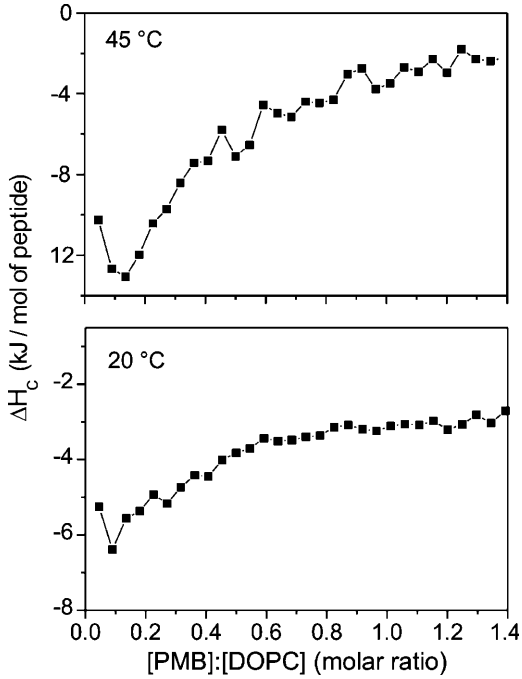

FIGURE 11 Enthalpy change of the DOPC-PMB reaction versus [PMB]/[DOPC] ratio at 20 and 45°C.

indicate, that in addition to a pure electrostatic binding, other interactions have to be considered. Srimal et al. (1996) and Thomas et al. (1999) presented similar data of the binding of PMB to different LPS and lipid A via ITC, but restricted to temperatures in the gel phase of the lipids. Therefore, their explanation of a general interaction mechanism, a binding process predominantly hydrophobic and entropy driven with little or no contribution from electrostatic interactions, does not hold true for physiological temperatures (see discussion below). The same group extended their measurements also to magainin analogs (Thomas et al., 2001) for which they described similar mechanisms.

In this work, we made a systematic investigation of the binding of polymyxins to endotoxins by varying: i), the measuring temperature, and ii), the length of the sugar chains of the used endotoxins. The data clearly illustrate that the PMB binding to lipid A and LPS with moderately long sugar-chain lengths (LPS Re to Rc) lead to a calorimetric endotherm below and an exotherm above the transition temperature T_c of the hydrocarbon chains (Figs. 2, 3, 8, and 9). This effect actually is phase specific, which is apparent from the data for lipid A ($T_c = 44$°C) as compared to LPS ($T_c = 30$–37°C for the different rough mutant LPS). The conversion of the endo- into an exotherm directly correlates with T_c (Figs. 3 *a* and 8). However, the results with the phospholipid DOPC, which is highly fluid at all temperatures, but for which exothermic signals are observed differing by a factor of two at 20 and 45°C (Fig. 11), also indicates an unspecific interaction of PMB with lipids that is temperature dependent and superimposes the binding process.

From the application of the Kirchoff equation we have calculated negative Δc_p for the binding of PMB to LPS in the gel phase as well as in the liquid crystalline phase. Due to the difficulties in obtaining accurate data for the interaction enthalpies, only estimates for Δc_p could be given. Δc_p(gel phase) ~ -4.3 kJ mol^{-1} K^{-1} and Δc_p(liquid crystalline phase) ~ -3.0 kJ mol^{-1} K^{-1} (± 1 kJ mol^{-1} K^{-1}). These values are high compared to Δc_p values obtained for the interaction of negatively charged phospholipids in the gel phase with divalent cations (Δc_p(gel) from ~ -0.1 to -0.6 kJ mol^{-1} K^{-1}). In the liquid crystalline phase the Δc_p for the phospholipid-divalent cation binding even becomes slightly positive (Garidel and Blume, 1999). Srimal et al. (1996) also found negative Δc_p values for the binding of LPS from *Escherichia coli* strains O11:B4 to PMB in the gel phase with Δc_p(gel) ~ -2.4 kJ mol^{-1} K^{-1}. These data are at least of the same order of magnitude as our Δc_p. Differences in Δc_p values can arise from different experimental conditions (e.g., buffer composition) and from the different LPS chemotypes (a smooth form LPS). Negative Δc_p values are interpreted as hydrophobic dehydration of LPS upon the formation of endotoxin-peptide complexes (more details with other lipids can be found by Garidel and Blume, 1999; Heerklotz and Epand, 2001; Yaminsky and Vogler, 2001; Machaidze et al., 2002; Uedaira et al., 2003).

As described, a different thermodynamical approach derived from the analysis of the temperature dependence of ΔG (Fig. 5) leads to positive c_p values. This apparent discrepancy may be explained by the fact that the addition of peptide leads to a phase change (fluidization) of the lipids, from which it might be implicated that the application of the Kirchoff equation is not necessarily possible. In future experiments, we will address this question by measuring over a much wider temperature range.

Binding of PMB to the endotoxin phosphates is much stronger than binding of the cations (J. Andrä, J. Howe, A. David, and K. Brandenburg, unpublished data), and leads to a rupture of the ordered assembly. This process is entropic in nature connected with an endothermic reaction; from our data, this reaction in the gel phase must be considerably higher than the exothermic reaction due to the electrostatic attraction between PMB and the phosphates. Similarly, the binding of divalent cations to glycerol-1-phosphoglycerol, the polar part of phosphatidylglycerol lipids, is endothermic and with increasing temperature the positive binding enthalpy increases (Garidel and Blume, 1999). Glycerol-1-phosphoglycerol and the divalent cations form ion pairs with a concomitant release of water. This is the reason for the positive Δc_p. Counterion adsorption on negatively charged surfaces will also lead to the release of water from the hydration shell of the polar headgroups and therefore positive Δc_p values. Dehydration of hydrophobic interfaces induced by electrostatic binding induce a negative Δc_p

(Garidel et al., 2000b) as found here for the endotoxin-peptide interactions The interaction of PMB with dodecanoic acid also has a positive Δc_p value as expected (Srimal et al., 1996).

It also has to be emphasized that the dynamic change of the acyl chain melting has to be considered leading to isothermal phase changes (Fig. 7 *B*). Already at a molar ratio [LPS]/[PMBN] = 1:0.4 the melting takes place in the lower temperature range from 10 to <30°C, i.e., the ITC measurement at 25°C (Fig. 3 *B*) occurs only at lower molar ratios in the pure gel phase. Thus, the low endotherm of PMBN as compared to PMB (Fig. 3 *A*) can be understood, because at this concentration and higher the LPS is more or less already in the liquid crystalline phase.

The finding that for LPS with long sugar chains (LPS Ra and S-form) no (Fig. 10 *A*) or only a small endotherm (Fig. 10 *B*) is observed at lower temperatures, indicates that the gel to liquid crystalline phase transition not always is connected with the endotherm-exotherm conversion of the binding reaction. This may be understood when considering the chemical structure of the LPS chemotypes (Fig. 1). Only for LPS Ra and S-form additional negative charged phosphate groups are present in the inner core region attached to the heptoses, which represent further target groups for PMB. Furthermore, the elongated sugar chains of LPS Ra and S-form represent a sterical barrier. Thus, a more complex mechanism than that observed for short sugar-chain endotoxins should take place. This may be directly connected with the observation that bacteria bearing LPS with shorter sugar chains (Re and Rd mutants) are more sensitive to the action of antimicrobial peptides than those with an LPS having a longer sugar chain (Ra mutant and wild types; Andrä et al., 2004). Similarly, the finding that the PMB-LPS binding stoichiometries, i.e., binding saturation, correspond to a pure charge compensation only for endotoxins with shorter sugar chain lengths, can be explained by a shielding effect of the sugar chains including the presence of additional charges in the core region as noted above, and by the immanent heterogeneity typical for wild-type (S-form) LPS. Generally, little is known about the detailed composition of S-form LPS. Only for some species such as *Salmonella abortus equi* an analysis has been published (Jiao et al., 1989). The authors found that the bioactive fraction within the heterogeneous LPS corresponded to a Ra or Rb mutant LPS with a complete lipid A moiety, whereas the fraction bearing the O-chain only had a reduced lipid A moiety with only 60% of the acyl chains compared to hexaacyl lipid A.

Summarized, the fact that the binding stoichiometries correspond to charge neutralization or are not far from this, emphasizes the importance of the electrostatic interaction as the main driving force of the endotoxin-PMB interaction. This interpretation is confirmed by the results for the acyl chain-lacking compound PMBN (Fig. 3 *B*), for which a direct hydrophobic interaction can be excluded.

The differences of the interaction of PMB with the bacterial outer membrane and isolated endotoxin aggregates must be emphasized. Hancock (1984) has proposed a self-promoted uptake of PMB into the bacterial cell. This was later corroborated by the findings that PMB induces transient permeability fluctuations in planar asymmetric phospholipid-LPS membranes (Schröder et al., 1992), which are large enough (2.4 nm) to allow the permeation of PMB (cross-section diameter 1.25 nm) through the outer membrane (Wiese et al., 1998). This means that in the natural outer membrane system PMB only transiently binds to the LPS monolayer, whereas the PMB binding to endotoxin aggregates leads to stable complexes.

To interpret the conversion from an endothermic binding reaction correlated with the gel phase and an exothermic binding reaction correlated with the liquid crystalline phase of the LPS hydrocarbon chains, it must be considered that the water- and cation-binding capacities of the two phases are different. In the gel phase, an ordered assembly of water molecules and hydrated counterions, mono- and divalent cations, bridge adjacent LPS molecules (Fig. 12 *A*; the term Me^+ indicates metal cations).

From DSC results peptide-lipid interactions can be classified into three types, leading to a specific signature in the heat capacity curve (Papahadjopoulos et al., 1975; Blume, 1991). a), Pure electrostatic interactions often lead to an increase in the phase transition enthalpy with a concomitant increase in the phase transition temperature. This is, for example, observed for the interaction of negatively charged phospholipids with divalent cations (Garidel and Blume, 1999). b), Electrostatic binding of compounds to the surface of a membrane, followed by partial penetration into the bilayer leads to more drastic changes of the phase transition enthalpy and temperature. The values are usually decreased due to the fact that acyl chain interactions are reduced. c), The interaction of integral proteins mainly is of pure hydrophobic nature, leading to a linear decrease of the phase transition enthalpy with increasing amounts of incorporated protein, a broadening of the phase transition and slight changes in the temperature maximum of the heat capacity curve.

According to this, situation ''b'' above seems to be dominant for the interaction of polymyxin B with endotoxin; as a driving force, electrostatic surface binding appears first, followed by a hydrophobic penetration. Comparing the interaction capacity of PMB and PMBN with endotoxins clearly shows that PMB is able to abolish the phase transition at peptide concentrations leading to charge neutralization, whereas much higher amounts of PMBN are required to induce a similar effect.

This interpretation that the electrostatic interaction is the primary driving force of the [LPS]-[PMB] binding becomes much more evident for LPS in its physiologically relevant liquid crystalline phase, in which the exothermic reaction due to the PMB-phosphate attraction predominates, because

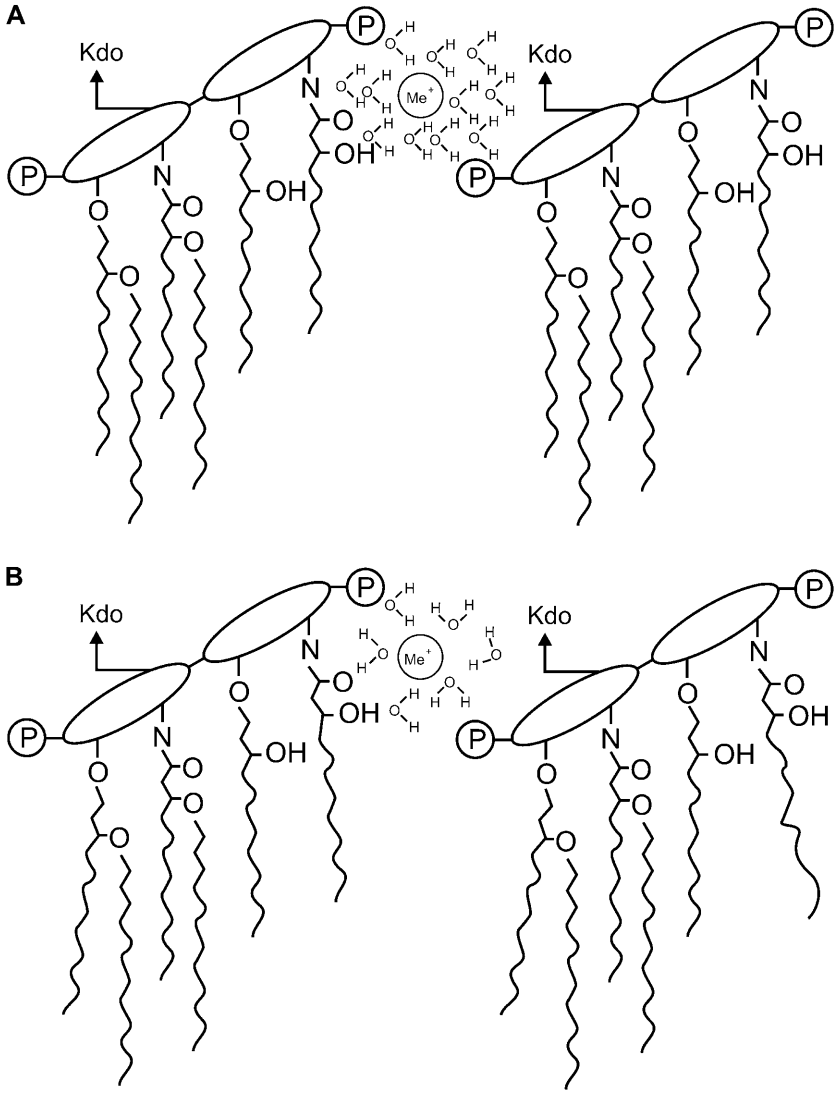

FIGURE 12 Schematic model of PMB binding to the LPS aggregates in the gel phase (*a*) and liquid crystalline phase (*b*). In the gel phase, an ordered network of water and cations bridge neighboring LPS molecules, whereas in the liquid crystalline phase this network is disrupted. The diglucosamine backbone of lipid A exhibits an inclination of 40–50° with respect to the membrane plane according to previous results (Seydel et al., 2000).

in this phase the ordered assembly of water and hydrated cations is much less expressed (Fig. 12 *B*).

For a quantitative determination of the different effects, the PMB binding of LPS Re may serve. For example, the addition of PMB to LPS at 24°C at a [PMB]/[LPS] molar ratio of 0.2 (Fig. 3 *A*) causes a $\Delta H_c \approx +60$ kJ/mol of PMB. Because at this ratio the absolute concentration of PMB is 0.03 mM, an absolute value of 1.8 J for the reaction can be calculated. The contribution of the (endothermic) acyl chain melting can be deduced from Fig. 6 and Table 1. Thus, at a [PMB]/[LPS] molar ratio of 0.2, the integrated peak intensity in Fig. 7 from 18 to 24°C would give 12 kJ/mol (the whole endotherm has 36 kJ/mol; Table 1). Considering the concentration of 1 mg/ml (0.4 mM) LPS, the calculation yields 4.8 J. This means that the contribution from the endothermic acyl chain melting has the same order of magnitude of the measured endotherm. This is in accordance with the interpretation of Koch et al. (1999) who proposed that at temperatures below T_c the ''phase transition'' of the LPS acyl chains induced by PMB would require more energy than provided by the binding of PMB to the negative charges. The enthalpic effect of dehydration of the metal cations in the gel phase can be estimated taking Mg^{2+} as an example. As the hydration enthalpy of Mg^{2+} amounts to 1920 kJ/mol (Atkins and de Paula, 2001), this would a give a value of 0.29 J for a 0.15 mM LPS dispersion. Because not all counterions consist of Mg^{2+} and because it is impossible to dehydrate Mg^{2+} completely, the actual value must be significantly lower. This means, that in the gel phase the binding of polymyxins to the lipid charges in a first step causes a disruption of the ordered water/cation layer in the LPS backbone. Subsequently, this leads to a melting of the hydrocarbon chains (fluidization) accompanied by a large endotherm.

A further potential contribution to the enthalpy change could result from reaggregation effects. However, in

accordance with earlier data (Brandenburg et al., 2002a,b) reaggregation of the LPS assembly into a multilamellar stack is enthalpically only of minor importance, as can be deduced from the PMB binding to the Mg^{2+} salt form of LPS Re, which itself forms a multilamellar aggregate (Fig. 6).

Our interpretation is strongly supported by investigations of the phase behavior of negatively charged or zwitterionic phospholipids in the presence of cations (Garidel et al., 2000a; Garidel and Blume, 1999). Thus, the latter authors found a much stronger interaction of divalent cations with dimyristoyl phosphatidylglycerol (DMPG) in the gel than in the liquid crystalline phase. In this phase, electrostatic binding was reduced as compared to the gel phase. The formation of a direct coordination complex was no longer possible. Furthermore, Garidel et al. (2000a) found that cation binding to DMPG induces a crystalline-like gel phase. This phase was connected with a deep penetration of the divalent cations into the polar headgroup region of DMPG, whereby the ester carbonyl groups were indirectly affected by strong hydrogen bonding of immobilized water molecules. In the liquid crystalline phase, in contrast, the interactions of all investigated cations (Mg^{2+}, Ca^{2+}, and Sr^{2+}) with the phospholipid were weak. Binder and Zschörnig (2002), who investigated the interaction of cations with differently hydrated 1-palmitoyl-2-oleoyl-phosphatidylcholine, found that the carbonyl groups were more accessible in the presence of divalent cations, because of their involvement in the hydration shell of the ions, pointing into the same direction.

From these data it becomes clear that in particular divalent cations are tightly bound to the lipids in the gel phase, and cause a much stronger hydrogen binding than in the liquid crystalline phase. This is consistent with the above interpretation, whereby PMB binding to the negatively charged LPS destroys the ordered ion/water system in the gel phase. Our interpretation is also supported by recent observations of Cheng et al. (2003), who investigated the ordering of water molecules between phospholipid bilayers. They found ordered water molecules close to the phospholipid bilayer surface, with the symmetry axis along the direction normal to the bilayer. The percentage of oriented molecules decreased with an increase in the interlamellar spacing, and was higher for negatively charged than for zwitterionic phospholipids.

Finally, for a pharmacological assessment of our findings, the saturation values of the peptide/LPS concentration ratios as found here ($\approx$ equimolar except for S-form LPS) are not far away from the ''physiological'' situation, taken form a rough estimate of the endotoxin concentrations in septic patients (μM) and the minimal inhibitory concentration values for PMB (also μM) (Morrison, 1996). The use of this peptide, however, is restricted in the case of systemic infections such as sepsis by its intrinsic cytotoxicity. Aside from this, the use of newly developed peptides depends on its metabolic fate, i.e., how long it remains in the circulation without being excreted.

We are indebted to G. von Busse for assistance in the ITC measurements. We kindly acknowledge the expert help of B. Fölting for performing the DSC.

This study has been carried out with financial support from the Commission of the European Communities under the specific RTD program ''Quality of Life and Management of Living Resources'', QLK2-CT-2002-01001, and ''Antimicrobial endotoxin neutralizing peptides to combat infectious diseases''.

REFERENCES

Alexander, C., and E. T. Rietschel. 2001. Bacterial lipopolysaccharides and innate immunity. *J. Endotoxin Res.* 7:167–202.

Andrä, J., M. H. J. Koch, R. Bartels, and K. Brandenburg. 2004. Biophysical characterization of endotoxin inactivation by NK-2, an antimicrobial peptide derived from mammalian NK-lysin. *Antimicrob. Agents Chemother.* 48:1593–1599.

Atkins, P., and J. de Paula. Physical Chemistry. 2001. Oxford University Press, Oxford, UK.

Binder, H., and O. Zschörnig. 2002. The effect of metal cations on the phase behaviour and hydration characteristics of phospholipid membranes. *Chem. Phys. Lipids.* 115:39–61.

Blume, A. 1991. Biological calorimetry: membranes. *Thermochim. Acta.* 193:299–347.

Blume, A., and P. Garidel. 1999. Lipid model membranes and biomembranes. *In* From Macromolecules to Man. R. B. Kemp, editor. Elsevier, Amsterdam, The Netherlands. 109–173.

Boulin, C., R. Kempf, M. H. J. Koch, and S. M. McLaughlin. 1986. Data appraisal, evaluation and display for synchrotron radiation experiments: hardware and software. *Nucl. Instrum. Methods.* A249:399–407.

Brade, H., S. M. Opal, S. N. Vogel, and D. C. Morrison. 1999. Endotoxin in Health and Disease. H. Brade, S. M. Opal, S. N. Vogel, and D. C. Morrison, editors. Marcel Dekker, New York, NY and Basel, Switzerland.

Brandenburg, K., M. D. Arraiza, G. Lehwark-Yvetot, I. Moriyon, and U. Zähringer. 2002a. The interaction of rough and smooth form lipopolysaccharides with polymyxins as studied by titration calorimetry. *Thermochim. Acta.* 394:53–61.

Brandenburg, K., I. Moriyon, M. D. Arraiza, G. Lehwark-Yvetot, M. H. J. Koch, and U. Seydel. 2002b. Biophysical investigations into the interaction of lipopolysaccharide with polymyxins. *Thermochim. Acta.* 382:189–198.

Brandenburg, K., W. Richter, M. H. J. Koch, H. W. Meyer, and U. Seydel. 1998. Characterization of the nonlamellar cubic and H_{II} structures of lipid A from *Salmonella enterica* serovar Minnesota by X-ray diffraction and freeze-fracture electron microscopy. *Chem. Phys. Lipids.* 91:53–69.

Brandenburg, K., and U. Seydel. 1990. Investigation into the fluidity of lipopolysaccharide and free lipid A membrane systems by Fourier-transform infrared spectroscopy and differential scanning calorimetry. *Eur. J. Biochem.* 191:229–236.

Carneiro, F. A., M. L. Bianconi, G. Weissmüller, F. Stauffer, and A. T. da Poian. 2002. Membrane recognition by vesicular stomatitis virus involves enthalpy-driven protein-lipid interactions. *J. Virol.* 76:3756–3764.

Cheng, J.-X., S. Pautot, D. A. Weitz, and X.-S. Xie. 2003. Ordering of water molecules between phospholipid bilayers visualized by coherent anti-Stokes Raman scattering microscopy. *Proc. Natl. Acad. Sci. USA.* 100:9826–9830.

Gabriel, A. 1977. Position-sensitive X-ray detector. *Rev. Sci. Instrum.* 48:1303–1305.

Galanos, C., B. Jiao, T. Komuro, M. A. Freudenberg, and O. Lüderitz. 1988. Large-scale fractionation of S-form lipopolysaccharide from

Salmonella abortus equi. Chemical and serological characterization of the fractions. *J. Chromatogr.* 440:397–404.

Galanos, C., O. Lüderitz, and O. Westphal. 1969. A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* 9:245–249.

Gallagher, K., and K. Sharp. 1998. Electrostatic contributions to heat capacity changes of DNA-ligand binding. *Biophys. J.* 75:769–776.

Garidel, P., and A. Blume. 1999. Interaction of alkaline earth cations with the negatively charged phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol: a differential scanning and isothermal titration calorimetric study. *Langmuir.* 15:5526–5534.

Garidel, P., A. Blume, and W. Hübner. 2000a. A Fourier transform infrared spectroscopic study of the interaction of alkaline earth cations with the negatively charged phospholipid 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol. *Biochim. Biophys. Acta.* 1466:245–259.

Garidel, P., A. Erbe, and A. Blume. 2000b. The thermodynamic characterization of pentalysin-lipid interactions studied by isothermal titration calorimetry. *J. Liposome Res.* 10:219–220.

Hancock, R. E. W. 1984. Alterations in outer membrane permeability. *Annu. Rev. Microbiol.* 38:237–264.

Hancock, R. E. W. 1997. Peptide antibiotics. *Lancet.* 349:418–422.

Heerklotz, H. H., and R. M. Epand. 2001. The enthalpy of acyl chain packing and the apparent water-accessible apolar surface area of phospholipids. *Biophys. J.* 80:271–279.

Jelesarov, I., and H. R. Bosshard. 1999. Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition. *J. Mol. Recognit.* 12:3–18.

Jiao, B., M. Freudenberg, and C. Galanos. 1989. Characterization of the lipid A component of genuine smooth-form lipopolysaccharide. *Eur. J. Biochem.* 180:515–518.

Jürgens, G., M. Müller, P. Garidel, M. H. J. Koch, H. Nakakubo, A. Blume, and K. Brandenburg. 2002. Investigation into the interaction of recombinant human serum albumin with Re-lipopolysaccharide and lipid A. *J. Endotoxin Res.* 8:115–126.

Koch, M. H. J., and J. Bordas. 1983. X-ray diffraction and scattering on disordered systems using synchrotron radiation. *Nucl. Instrum. Methods.* 208:461–469.

Koch, P.-J., J. Frank, J. Schüler, C. Kahle, and H. Bradaczek. 1999. Thermodynamics and structural studies of the interaction of polymyxin B with deep rough mutant lipopolysaccharides. *J. Colloid Interface Sci.* 213:557–564.

Kubesch, P., J. Boggs, L. Luciano, G. Maass, and B. Tümmler. 1987. Interaction of polymyxin B nonapeptide with anionic phospholipids. *Biochemistry.* 26:2139–2149.

Loladze, V. V., D. N. Ermolenko, and G. I. Makhatadze. 2001. Heat capacity changes upon burial of polar and nonpolar groups in proteins. *Protein Sci.* 10:1343–1352.

Machaidze, G., A. Ziegler, and J. Seelig. 2002. Specific binding of Ro 09–0198 (cinnamycin) to phosphatidylethanolamine. A thermodynamic analysis. *Biochemistry.* 41:1965–1971.

Mamat, U., U. Seydel, O. Holst, D. Grimmecke, and E. T. Rietschel. 1999. Lipopolysaccharides. *In* Carbohydrates and Their Derivatives Including Tannins, Cellulose, and Related Lignins. B. M. Pinto, editor. Elsevier Science, Amsterdam, The Netherlands. 179–240.

Morrison, D. C. (Ed.) 1996. Antibiotic-mediated endotoxin release: contribution to pathogenesis in Gram-negative sepsis. *J. Endotoxin Res.* 3.

Papahadjopoulos, D., M. Moscarello, E. H. Eylar, and T. Isac. 1975. Effects of proteins on thermotropic phase transitions of phospholipid membranes. *Biochim. Biophys. Acta.* 401:317–335.

Schindler, M., and M. J. Osborn. 1979. Interaction of divalent cations and polymyxin B with lipopolysaccharide. *Biochemistry.* 18:4425–4430.

Schröder, G., K. Brandenburg, and U. Seydel. 1992. Polymyxin B induces transient permeability fluctuations in asymmetric planar lipopolysaccharide/phospholipid bilayers. *Biochemistry.* 31:631–638.

Scott, M. G., and R. E. W. Hancock. 2000. Cationic antimicrobial peptides and their multifunctional role in the immune system. *Crit. Rev. Immunol.* 20:407–431.

Seydel, U., M. Oikawa, K. Fukase, S. Kusumoto, and K. Brandenburg. 2000. Intrinsic conformation of lipid A is responsible for agonistic and antagonistic activity. *Eur. J. Biochem.* 267:3032–3039.

Srimal, S., N. Surolia, S. Balasubramanian, and A. Surolia. 1996. Titration calorimetric studies to elucidate the specificity of the interactions of polymyxin B with lipopolysaccharides and lipid A. *Biochem. J.* 315:679–686.

Thomas, C. J., and A. Surolia. 1999. Kinetics of the interaction of endotoxin with polymyxin B and its analogs: a surface plasmon resonance analysis. *FEBS Lett.* 445:420–424.

Thomas, C. J., N. Surolia, and A. Surolia. 1999. Surface plasmon resonance studies resolve the enigmatic endotoxin neutralizing activity of polymyxin B. *J. Biol. Chem.* 274:29624–29627.

Thomas, C. J., N. Surolia, and A. Surolia. 2001. Kinetic and thermodynamic analysis of the interactions of 23-residue peptides with endotoxin. *J. Biol. Chem.* 176:35701–35706.

Uedaira, H., H. Kono, P. Ponraj, K. Kitajima, and A. Sarai. 2003. Structure-thermodynamic relationship in protein-DNA binding: heat capacity changes. *Genome Informatics.* 14:510–511.

Vaara, M. 1992. Agents that increase the permeability of the outer membrane. *Microbiol. Rev.* 56:395–411.

Westphal, O., O. Lüderitz, and F. Bister. 1952. Über die Extraktion von Bakterien mit Phenol/Wasser. *Z. Naturf.* [in German] 7:148–155.

Wiese, A., M. Münstermann, T. Gutsmann, B. Lindner, K. Kawahara, U. Zähringer, and U. Seydel. 1998. Molecular mechanisms of polymyxin B-membrane interactions: direct correlation between surface charge density and self-promoted uptake. *J. Membr. Biol.* 162:127–138.

Yaminsky, V. V., and E. A. Vogler. 2001. Hydrophobic hydration. *Curr. Opin. Colloid Interface Sci.* 6:342–349.

Zähringer, U., B. Lindner, and E. T. Rietschel. 1999. Chemical structure of lipid A. Recent advances in structrural analysis of a biologically active molecules. *In* Endotoxin in Health and Disease. H. Brade, S. M. Opal, S. N. Vogel, and D. C. Morrison, editors. Marcel Dekker, New York. 93–114.