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The interaction of rough and smooth form lipopolysaccharides with polymyxins as studied by titration calorimetry

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Abstract

The interaction of amphiphilic lipopolysaccharides (LPS, endotoxin) from Gram-negative bacteria with the polycationic decapeptide polymyxin B (PMB) and its non-apeptide PMBN was studied mainly by applying isothermal titration calorimetry (ITC). The LPS investigated comprise various mutant and wild-type forms from *Salmonella minnesota*, differing in the length of their sugar chain, and some wild-type LPS from other strains. It can be shown that three effects occur after binding of the peptides to LPS, an electrostatic attraction of the positive charges of the polymyxins to the negative charges in particular in or near the lipid backbone of LPS (termed lipid A) leading to a calorimetric exotherm, a reorientation of the LPS aggregate structure, and a fluidization of the LPS acyl chains which are connected with endothermic processes. The superposition of these three effects leads to sometimes very complex titration curves, apparently due to the presence of further negative charges distal to lipid A. The results from the ITC experiments allow the determination of the binding stoichiometry—which is important for the adjustment of dosages in therapeutical applications—lying at a molar ratio [PMB]:[LPS] of 1:1.5 for LPS with shorter sugar chains and 1:2 to 1:3 for those with longer sugar chains (wild-types).

Keywords: Lipopolysaccharide; Isothermal titration calorimetry; Peptide antibiotics; Sepsis

1. Introduction

Lipopolysaccharides (LPS) are located as main amphiphilic component on the outer leaflet of the outer membrane of Gram-negative bacteria, but can be released into the environment during cell division, and various biochemical processes and by the attack of the host's immune system. LPS is termed endotoxin due to their ability to induce a variety of biological effects in mammals, in particular the production of proinflammatory cytokines such as tumor-necrosis-factor-α (TNF-α) and interleukins [1]. The production of these mediators at low endotoxin concentrations may lead to beneficial biological effects, at higher concentrations, however, the release of cytokines leads to self-poisoning of the body, eventually resulting in the shock syndrom [2].

LPS is comprised of a sugar portion with a varying length from polysaccharide- (the O-antigen) to oligosaccharide chains depending on the bacterial species or bacterial mutants—smooth form LPS to rough mutant LPS Re to Ra—which is covalently linked to the hydrophobic moiety of LPS, lipid A, anchoring the LPS molecule into the membrane. Lipid A

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is composed of a β(1 → 6)-interlinked diglucosamine backbone which is phosphorylated in positions 1 and 4′ and acylated by 6–7 partially hydroxylated hydrocarbon chains in ester- and amide-linkage to positions 3′, 3 and 2′, 2 for enterobacterial strains [3]. For LPS from other bacteria, the lipid A structure may differ in particular with respect to the acylation pattern. For example, *Yersinia pestis* lipid A consists of a mixture of essentially tetraacyl- and hexaacyl-lipid A [4]. For nearly all LPS known so far, two very unusual sugars, 2-keto-3-deoxyoctonate (Kdo), are linked to lipid A each one having negative charge in the carboxylate group at neutral pH. Thus, LPS carries a high number of negative charges. From these considerations it may not be suprising that polycationic drugs (peptides, proteins, and/or antibiotics) such as bacteria-derived polymyxin B (PMB) and its non apeptide PMBN as well as various endogenous cationic proteins such as lactoferrin, the defensins, the CAP family, and bactericidal permeability-increasing protein (BPI), [5] were found to effectively protect against the pathophysiological effects of LPS. Although a variety of studies describes the effects of PMB on outer membranes of intact bacteria and on isolated LPS or anionic phospholipids [6,7], the systematic therapeutic application of PMB is severely hampered by its toxicity at higher concentrations. It has been found that PMBN—a deacylated derivative of PMB—is essentially non-toxic and also devoid of bactericidal potency, but still retains LPS-binding activity. PMBN was also found to sensitize various pathogenic bacterial strains against the direct bactericidal effect of human serum [8].

For a detailed study of the interaction of drugs with target structures such as LPS, the application of a variety of physical techniques may be helpful for the evaluation of the mechanisms involved in removal of pro-inflammatory structures. One of the techniques is isothermal titration calorimetry (ITC) allowing the determination of binding constants and binding stoichiometry. We have recently observed that the ITC curves of the binding of PMB and PMBN to LPS with long sugar chains could readily be interpreted by the electrostatic attraction of the positive charges of the peptides with the negatively charged groups of LPS [9]. For short sugar chain LPS and lipid A, however, very complex titration curves were observed, which could be interpreted by the superposition of three effects, the electrostatic interaction, the fluidization of the LPS acyl chains, and the reaggregation from a cubic into a multilamellar structure due to peptide binding.

In the present paper, a systematic analysis of polymyxin binding to LPS with varying lengths of the sugar chains, i.e. from various rough mutant and wild-type strains by ITC experiments is presented.

2. Materials and methods

2.1. Lipopolysaccharides

LPS from the rough mutants Rd1 (strains R7, $M = 2950$ Da, and Rz, $M = 3100$ Da), Rc (strain R5, $M = 3100$ Da), Rb (strain R345, $M = 4020$ Da), Ra (strain R60, $M = 4300$ Da) from *S. minnesota* were extracted from bacteria grown at 37 °C by the phenol/chloroform/petrol ether method [10]. Wild-type LPS from smooth strains *S. minnesota*, *Salmonella abortus equi*, and *Escherichia coli* serotype O8:K127 were extracted by the phenol:water procedure [11]. LPS from *Y. pestis* was isolated from bacteria grown in standard tryptic soy broth [4]. After extraction all LPS were purified and lyophilized.

The chemical structures of rough mutant LPS from *S. minnesota* are given in Fig. 1. The different fractions of the wild-type LPS were analysed by gel electrophoresis using polyacrylamide as carrier substrate (PAGE) and sodium dodecyl sulfate (SDS) as agent which leads to a negative charge of all fractions and inhibits their aggregation. The different fractions were made visible by silver staining with a final concentration of 1.3% $AgNO_3$. The chemical structure of LPS from *Y. pestis* is not completely elucidated. It is known, however, that it has no O-antigen, and by SDS–PAGE analysis the molecular weight can be estimated to lie around 4500.

2.2. Sample preparation

The LPS samples were usually prepared as aqueous dispersions at high buffer (20 mM Hepes) content, depending on the sensitivity of the technique: 0.1 mM for the ITC experiment, 5 mM for the infrared, and 40 mM for the X-ray experiment. In all cases, the lipids were suspended directly in Hepes buffer and

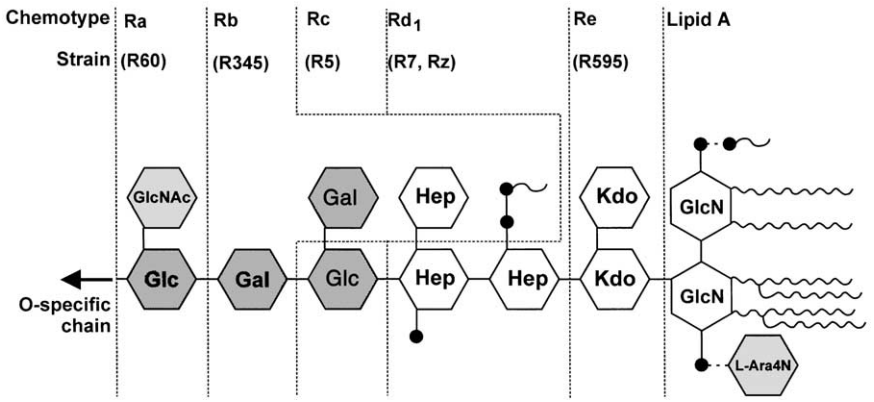

Fig. 1. Schematic structure of various LPS rough mutant LPS from *S. minnesota*. The O-specific polysaccharide consists of a varying number of repeating units of a pentasaccharide. Abbreviations: Hep, L-glycero-D-*manno*-heptose; Gal, galactose; Glc, glucose; GlcN, glucosamine; GlcNAc, *N*-acetyl glucosamine; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; L-Ara4N, 4-amino-4-deoxy-L-arabinose; (●) phosphate. Beside the two phosphate groups in the lipid A backbone, further phosphate or pyrophosphoethanolamine groups are present in the heptose region for the strains Rz, R5, R345, and R60; (– – –) indicates non-stoichiometric substitution.

temperature-cycled several times betwen 5 and 70 °C and then stored at least 12 h before measurement.

2.3. Isothermal titration calorimetry

Microcalorimetric experiments of peptide binding to endotoxins were performed on a MCS isothermal titration calorimeter (Microcal Inc., Northampton, MA, USA) at 37 °C. Endotoxin samples (0.1 mM) were filled into the microcalorimetric cell (volume 1.3 ml) and the peptides at a concentration of 1–3 mM into the syringe compartment (volume 100 μl), each after thorough degassing of the suspensions. After temperature equilibration, the peptides were titrated in 3 μl portions every 10 min into the endotoxin-containing cell, which was stirred constantly. The enthalpy change after each injection measured by the ITC instrument was plotted versus time. As a control, the peptide was titrated into pure Hepes solution. This exothermic reaction due to dilution turned out to be negligible. The total heat signal from each experiment was determined as the area (integral) under the respective single peaks and plotted versus the [peptide]:[endotoxin] molar ratio. Since, the instrument works in temperature equilibrium at a constant 'current feedback' corresponding to a power of approximately 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 curves were repeated at least four times.

2.4. FTIR spectroscopy and X-ray diffraction

The infrared spectroscopic measurements were performed on an FTIR spectrometer IFS-55 essentially as described [9]. For the determination of the order of the lipid acyl chains the peak position of the symmetric stretching vibration of the methylene groups was evaluated [12]. X-ray diffraction measurements were performed at the European Molecular Biology Laboratory (EMBL) outstation at the Hamburg synchrotron radiation facility HASYLAB using the double-focusing monochromator-mirror camera X33 as described [13]. Briefly, diffraction patterns in the range of the scattering vector $0.07 < s < 1\,\mathrm{nm}^{-1}$ ($S = 2\sin(\theta)/\lambda, 2\theta$) is the scattering angle and λ the wavelength = 0.15 nm) were recorded at 40 °C with exposure times of 2 or 3 min using a linear detector with delay line readout. In the diffraction pattern presented here, the logarithm of the diffraction intensities $\log I(s)$ is plotted against the scattering vector s.

3. Results and discussion

Since wild-type LPS are heterogenous with respect to the length of their sugar chains, various

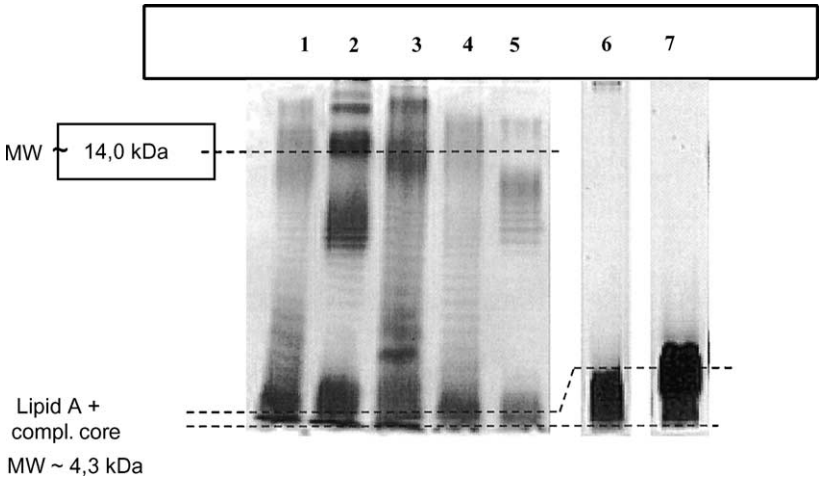

Fig. 2. Wild-type *S*-form LPS (lanes 1–5), mutant Re LPS (lane 6), and O-antigen lacking naturally *R*-LPS (lane 7) analysed by SDS–PAGE followed by periodate-silver staining using 10% (lanes 1–5) or 18% (lanes 6 and 7) polyacrylamide gels. Two micrograms of LPS from the following bacteria were loaded: (1) *S. minnesota*; (2) *E. coli* O8:K127; (3) *S. abortus equi*; (4) *Pseudomonas aeruginosa* F2; (5) *S. minnesota* Re mutant; (6) *Y. pestis* Kim. The ladder-like banding patterns indicate the heterogeneity of the samples with the highest molecular weights on top and the lowest (lipid A plus complete core oligosaccharide) at the bottom. Note that the lipid A core appears as a flat or broad band depending upon the percentage amount of the polyacrylamide in the gel.

S- and *R*-form LPS were analysed by SDS–PAGE and silver-staining. The results are shown in Fig. 2. Clearly, the ladder-like structures indicate the occurrence of a multiple of saccharide repeating units forming LPS molecules of various chain lengths and molecular weights. Assuming lipid A plus complete oligosaccharide core to correspond to approximately 4100 Da (lower rim), LPS from *S. minnesota* and *E.coli* express a maximum at 14,000 Da, while that for LPS from *S. abortus equi* is slightly lower. Considering the overall heterogeneity, a molecular weight of 10,000 Da for all *S*-form LPS was tentatively assumed for the ITC experiments. The same analyses (Fig. 2) show that *Y. pestis* Kim LPS is devoid of the O-polysaccharide and has a molecular weight higher than that of the *S. minnesota* Re mutant (i.e. in the 4000–5000 Da range).

A typical isothermal titration of LPS with PMB is given in Fig. 3a for LPS from *S. abortus equi*. The addition of PMB (3 μl in the range 2–4 mM) was done every 5 min to 0.1 mM LPS. A strong exothermic reaction is observed which can be interpreteted as resulting from the electrostatic attraction between the five positive charges of PMB and the negative charges (four to six) of *S*-form LPS. Additionally, during the time interval 70–120 min a weak endothermic reaction is superimposed. The plot of the enthalpy change versus molar ratio of PMB to LPS (Fig. 3b) shows that saturation of binding takes place a first time at a molar ratio of 2.2:2.3 and then at a ratio of four PMB molecules per one LPS molecule. This suggests that LPS subspecies may exist with a different number of negative charges. These subspecies could possibly correspond to the two maxima in the gel of LPS from *S. abortus equi* (lane 3 in Fig. 2). However, since the LPS are in an aggregated state, their negative charges may not readily be accessible for the peptide thus, explaining the observation that for binding saturation much more peptide molecules are necessary than corresponding to a pure charge compensation.

Due to earlier findings [9] that electrostatic interaction, acyl chain fluidization and reaggregation superimpose, also the latter two parameters were monitored. In Fig. 4, the $\beta \leftrightarrow \alpha$ gel to liquid crystalline phase behaviour of LPS from *S. abortus equi* at different PMB concentrations is given, showing a strong fluidization of the acyl chains. At 37 °C, the wavenumber values increase by more than 1 cm^{-1}. The X-ray small-angle diffraction patterns are given in Fig. 5 for LPS from *S. abortus equi* in the absence (a) and presence (b) of PMB. It is found that even at this very high PMB concentration nearly no change of the basic pattern,

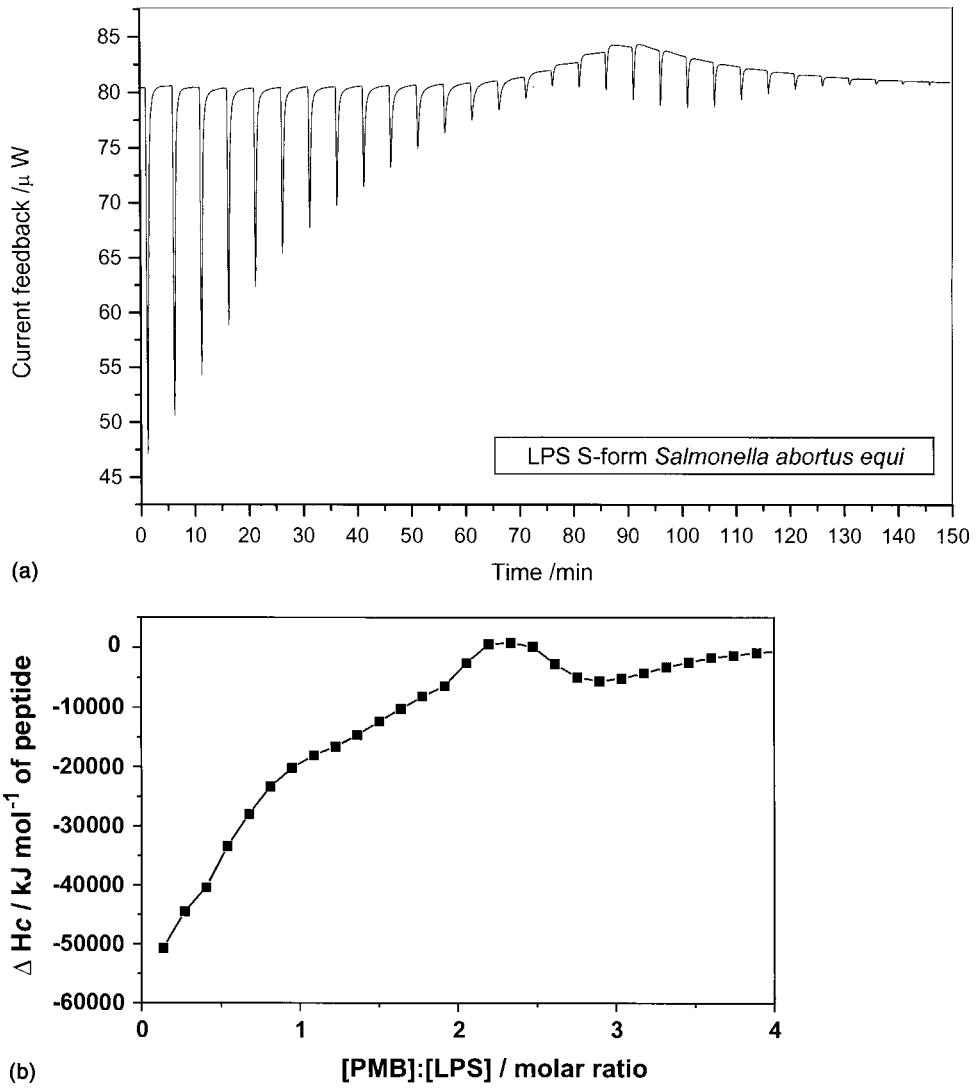

Fig. 3. (a) Isothermal calorimetric titration of wild-type LPS from *S. abortus equi* (0.1 mM) with PMB (5 mM). The 1.3 ml dispersion of LPS in Hepes buffer in the calorimetric cell was titrated every 5 min with 3 μl of the peptide. The reduction of the current feedback indicates an exothermic reaction. (b) Enthalpy change of the LPS-peptide reaction vs. [PMB]:[LPS] molar ratio for LPS from *S. abortus equi* from calorimetric titration as shown in (a). Binding saturation is found around a molar ratio of 2.4 and >4 indicating the existence of subspecies with differently long sugar chains.

which corresponds to a unilamellar structure [13] takes place. Therefore, the dominating effect in the ITC curves for smooth form LPS is the electrostatic attraction. No effect due to reaggregation as found earlier for LPS with much shorter sugar chains [9] can be assumed, and despite a strong fluidization, the enthalpy change due to this effect can be neglected.

The results for ITC experiments with LPS from *Y. pestis* and the two polymyxins (Fig. 6) indicate for PMB two binding places. Furthermore, the binding saturation values (∼1.4 for PMB and >2.0 for PMBN) indicate again that not all negative charges of LPS may be accessible for the peptides, since this LPS has four negative, while PMB has five and PMBN

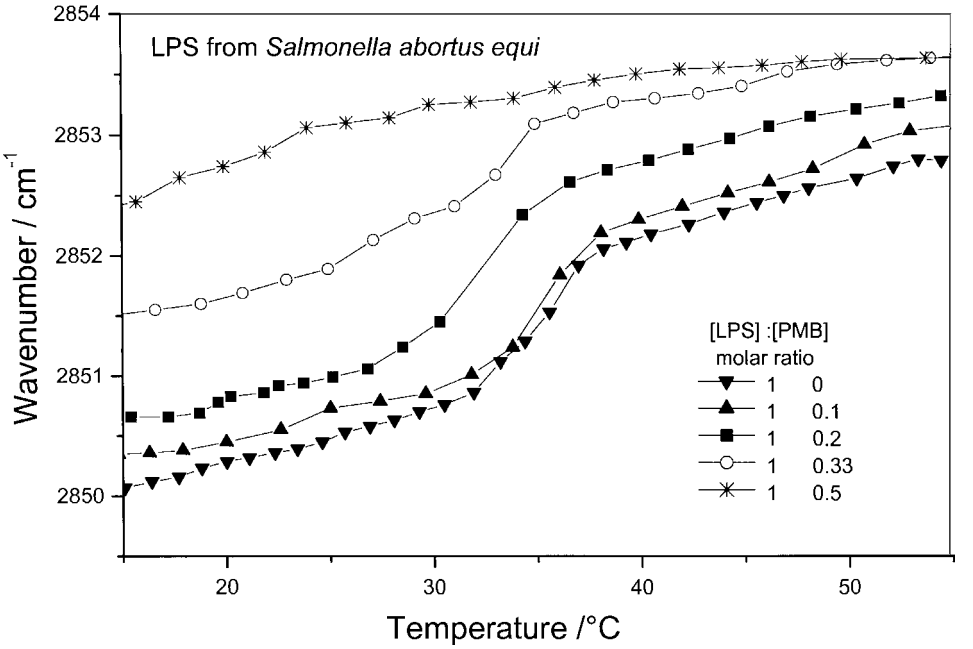

Fig. 4. Gel to liquid crystalline ($\beta \leftrightarrow \alpha$) phase transition of the acyl chains of LPS from *S. abortus equi* at different [LPS]:[PMB] molar ratios, determined infrared-spectroscopically from the peak position of the symmetric stretching vibration of the methylene groups ranging from 2850 cm^{-1} in the gel to 2852–2853 cm^{-1} in the liquid crystalline phase.

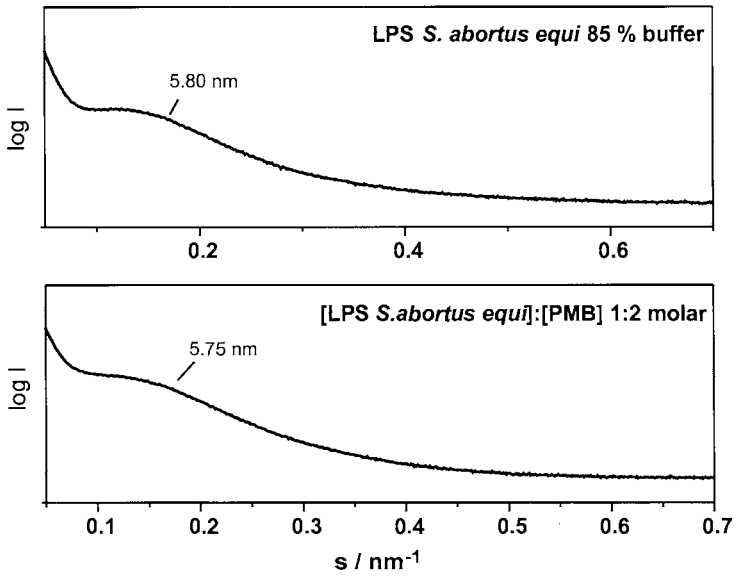

Fig. 5. Synchrotron radiation X-ray diffraction of LPS from *S. abortus equi* in buffer (a) and at [LPS]:[PMB] = 1:2 molar ratio (b) at 90% water content and 37 °C. Presented is the logarithm of the scattering intensity $\log I$ vs. the 'scattering vector' $s = 2 \sin\theta/\lambda$; where θ is the scattering angle, λ: wavelength = 0.15 nm.

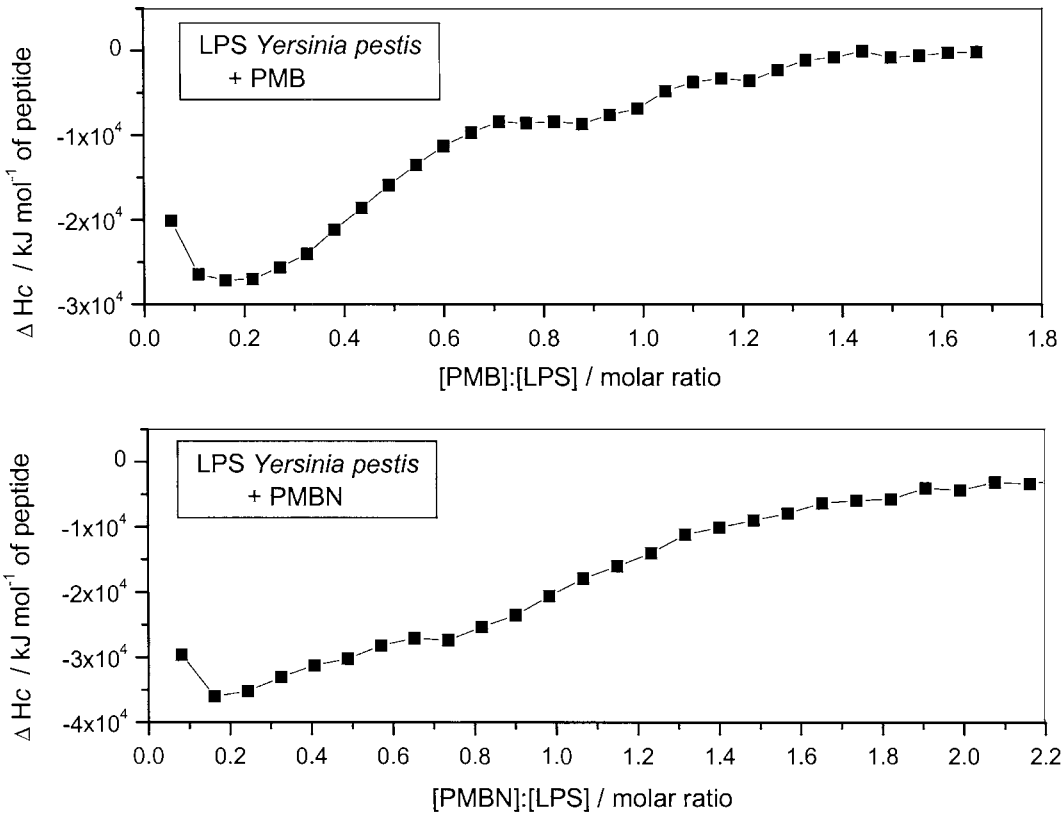

Fig. 6. Enthalpy change of the LPS-peptide reaction vs. [peptide]:[LPS] molar ratio for PMB and its non apeptide PMBN and LPS from *Y. pestis* from calorimetric titration. The [peptide]:[LPS] saturation values indicate that beyond charge compensation still a reaction takes place.

four positive charges. Therefore, charge compensation should already occur at [PMB]:[LPS] = 0.8 and [PMBN]:[LPS] = 1.0.

Finally, ITC experiments for different mutants and smooth form from *S. minnesota* were performed (Fig. 7). Interestingly, for the two Rd1 mutant LPS saturation takes place at similar values, although LPS Rz has two further negative charges in the heptose region (see Fig. 1), which are not present for LPS R7. Furthermore, for LPS Rz (weak) and for LPS R5 (strong) an endotherm is superimposed between [PMB]:[LPS] = 0.25 and 0.7, which may be due to a reaggregation of the LPS assembly as discussed above. These findings will give rise to further investigations on the aggregate structures.

With respect to the fluidization effect of PMB, the data for the different rough mutant LPS indicate nearly identical increase of the wavenumber values at 37 °C due to peptide binding in the range of 1 cm^{-1} (Fig. 4 and data not shown). As stated above, however, this effect contributes only negligibly to the enthalpic change observed in the ITC experiments.

The comparison of the saturation values (binding stoichiometry) for the different LPS (Fig. 7) shows a clear increase of the [PMB]:[LPS] molar ratios with increasing sugar chain length. From these data it can be concluded that not only the negatively charged phosphate and carboxylate groups in or proximal to the lipid A backbone, but also charges in the core oligosaccharide, may be binding-sites for PMB.

The findings of the present investigation are in accordance with the data presented by Koch et al. [14], who found—also with ITC—a strong exotherm (60 kJ/mol), and who proposed as first step of binding an electrostatic association and as second step penetration of PMB into the LPS membrane. In contrast

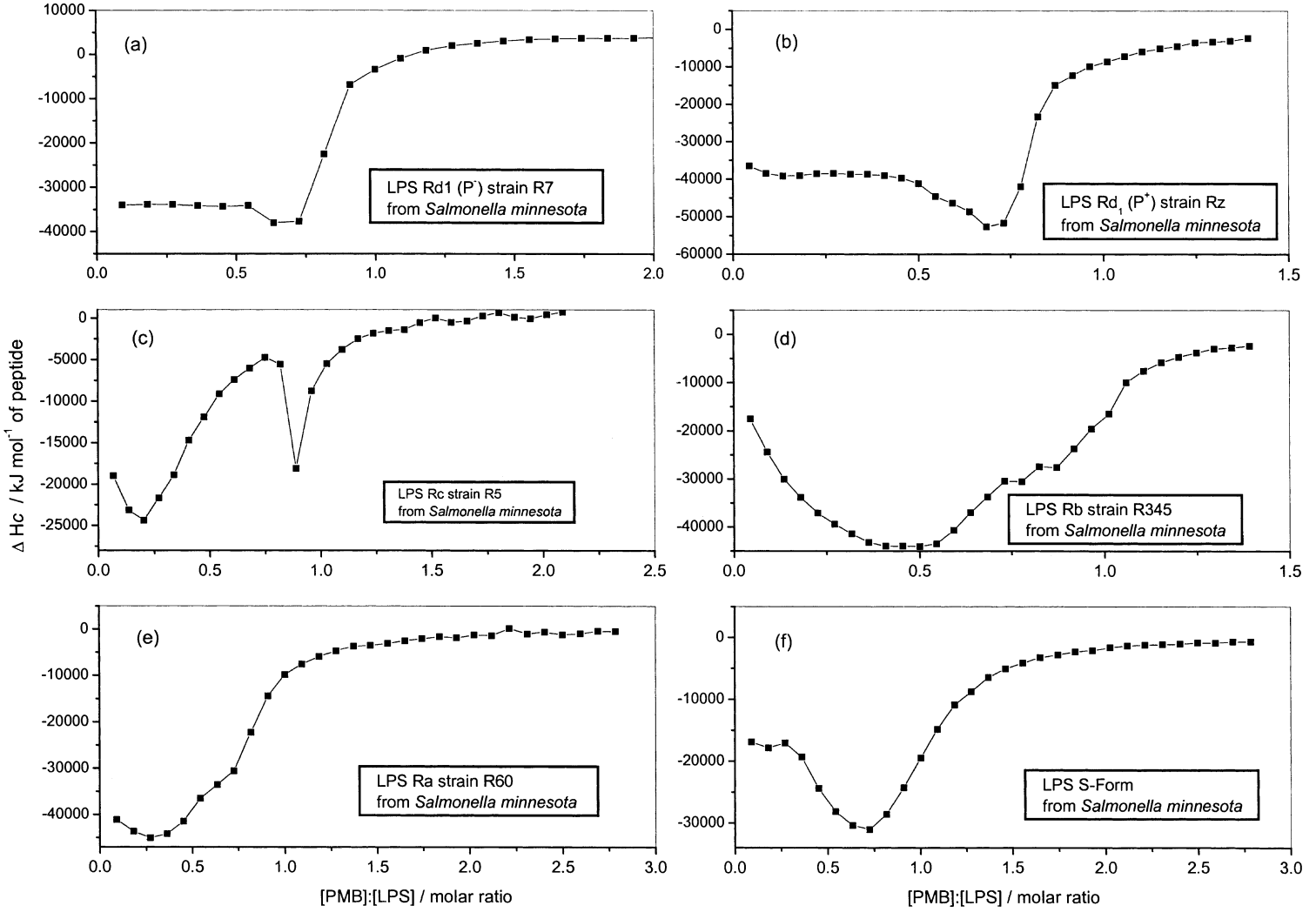

Fig. 7. Enthalpy changes of the LPS-peptide reaction vs. [PMB]:[LPS] molar ratio for various rough mutant and wild-type LPS from *S. minnesota* from calorimetric titration. The sugar chain lengths increase from (a) to (f).

to our findings and to those of Koch et al. are the results published by the group of Surolia and coworkers [15,16] with PMB and related peptides and a series of different lipid A and LPS structures. The authors observed endothermic titration curves from which the binding of the peptide to the endotoxins was proposed to be a non-cooperative process with predominantly hydrophobic interaction as driving force, whereas the electrostatic interaction was assumed to play only a minor role. An explanation for the apparent discrepancy may be the measuring temperatures, since these authors performed their ITC measurements at non-physiological temperatures in the range 6–25 °C for LPS and 10–31 °C for lipid A which is significantly below the T_c for the respective samples. Therefore, their interpretation does not seem to be relevant with respect to the physiological situation. Also, a comparison is hampered by the use of a different, especially phosphate-containing buffer (50 mM sodium phosphate buffer), Furthermore, the fact that in PMB-resistant bacterial mutants the negative charges are compensated by an increasing amount of positive charges within the headgroup region of LPS emphasizes the importance of the electrostatic interaction [17].

Independently of the physiological relevance, the finding that above T_c the ITC curves are strictly exotherm and below T_c strictly endotherm indicates that the thermodynamics of the interaction differs. It was found that the binding of alkaline earth ions (Mg^{2+}, Ca^{2+} and Ba^{2+}) to negatively charged phospholipids is entropically driven and an endothermic process, since the ordered water molecule network is disrupted by the cations [18]. This process would correspond to what could take place below T_c in the gel phase of the LPS which is in accordance to the data of the Surolia and coworkers [15,16]. In the liquid crystalline phase of LPS as described here, the interaction process is enthalpic-driven and exothermic in accordance to the presented data. This should result from the fact that the cross-section of the lipid backbone is much higher in the liquid than in the gel phase and that the bound water molecules are much more unordered thus allowing the peptides directly to bind to the negatively charged groups in an exothermic reaction. For a nearer characterization of these processes, we are now performing a systematic study of the temperature dependence of the ITC curves of various LPS and lipid A structures with PMB and PMBN.

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