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Calorimetric investigations of the effect of polymyxin B on different Gram-negative bacteria

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Abstract

Biocalorimetry is an important tool to measure the metabolic effects of various bioactive agents on living bacteria. In this study, we examined the heat production of different bacterial strains after treatment with the standard antimicrobial peptide polymyxin B (PMB). We found, that the total amount of produced heat differs for bacterial strains, which have different sensitivity against the antimicrobial peptide. The heat production of sensitive strains like *Salmonella minnesota* R595 is 108 kJ/mol PMB, whereas the heat production of the PMB-resistant strain *Proteus mirabilis* R45 is 57 kJ/mol, i.e., only the half. The heat productions of other strains from *S. minnesota* lie in between these strains. The calorimetric data suggest that PMB interaction increases with increasing sensitivity of the bacteria against the drug. One explanation could be the different chemical structure of the lipopolysaccharide of the bacteria membrane, responsible for the PMB binding reaction.

Keywords: Gram-negative bacteria; Bacterial resistance; Polymyxin B; Heat production; Isothermal titration calorimetry

1. Introduction

Bacterial resistance is a major problem in medical critical care units, due to severe infections and the fact that conventional antibiotics may have only slight effects on some pathogenic bacteria [1]. The effects are not only dependent on the type of antibiotic, but also on a sufficiently high concentration of it, because the sensitivity of the bacteria may vary widely.

It has been shown that antimicrobial cationic peptides play an important role in host defence against microbial infections [2]. A well-characterized peptide antibiotic is the polycationic polymyxin B (PMB), a deca-peptide with a small hydrocarbon chain and five positive net charges. Although PMB is toxic for human cells at high concentrations [3], the lack of new antibiotics has lead to a renewed interest in this peptide. PMB belongs to the family of Polymyxins discovered 1947 in *Bacillus polymyxa* [4]. Only two of these peptides, polymyxin B and E, the latter also known as colistin, have been used as therapeuticals for humans. As recently reviewed by Falagas and Kasiakou [5], who found evidence that the toxicity of PMB is lower than previously reported, it is still discussed as a potential peptide antibiotic for clinical use. PMB is not only of interest because of its antimicrobial properties, but also on its very important ability to bind released bacterial pathogenicity factors such as endotoxins. Endotoxins, which are chemically lipopolysaccharides (LPS) are the main amphiphilic components of the outer leaflet of the outer membrane of Gram-negative bacteria such as *Escherichia coli*, *Salmonella* spp., *Vibrio cholerae*, and *Yersinis pestis*. LPS is an amphiphilic glycolipid with a membrane anchor, called lipid A, and a hydrophilic sugar moiety that consists of a variable sugar chain. LPS is released from the bacterial cells due to the action of the immune system or the action of antibiotics leading to the production of cytokines in immune cells. At too high concentrations of LPS, the overwhelming production of this inflammatory messenger can lead to multiorgan failure and the septic chock syndrome [6]. It was found that PMB conjugated with cytokine-binding proteins looses its toxicity and still acts as an endotoxin-binding peptide [7]. Thus, an effective antimicrobial peptide must kill bacteria as well as neutralize LPS.

The biological properties of PMB are well-described, it is used as a standard for the efficiency of newly developed peptide antibiotics. Furthermore, the mode of interaction of PMB with lipid membrane model systems was well investigated by biophysical means, which started three decades ago [8]. Moreover, PMB shows a rapid binding to Gram-negative bacteria

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[9] and induces fluctuations of the electric potential of their outer membrane by inducing lesions in it. Through those Lesion PMB can pass and fullfill the so-called "self-promoted uptake" [10].

In this study, we used well-characterized strains of the bacteria *Salmonella minnesota* and *E. coli*, with differences in PMB-sensitivity, to investigate the heat production effects on application of PMB in relation to the concentration of PMB needed to kill the bacteria. Furthermore, we also used the deep rough mutant strain R45 from *Proteus mirabilis*, a PMB-resistant strain. We try to connect our calorimetric data of the resistant and sensitive bacteria to the different chemical structures. In particular, the increase of positively charged arabinose sugars in the head group region leads to a variation in the different susceptibility [11].

Calorimetric investigations of bacteria started with the calorimetric characterization of the different phases of bacterial growth [12], and is at present a standard method to find the optimum condition for bacterial growth [13]. It has been shown for *Salmonella* bacteria, that the resistant pmrA strains only absorb about 25% of the PMB as compared to the sensitive parent strains [14], but no calorimetric data are available. To investigate the thermal reaction of the bacteria on binding to PMB, we used an isothermal titration calorimetry (ITC), working at 37 °C. We measured the total heat production of the reaction of PMB with different bacterial strains, and we were able to differentiate between bacteria with a different susceptibility against the antimicrobial peptide.

2. Experimental

2.1. Reagents

Polymyxin B was purchased from Sigma Chemicals (Deisenhofen, Germany), LB-medium was from Merck KgaA (Darmstadt, Germany), PBS-buffer from Biochrome AG (Berlin, Germany), and 96-well plates were from Greiner bio-one (Kremsmünster, Austria). The chemical structure of PMB is shown in Fig. 1a.

2.2. Preparation of bacterial cultures

Bacteria were obtained by suspending bacteria in LB-medium and leaving them overnight in culture. They were stored at 37 °C and were shaken at 140 turns per minute. When the culture was in the stationary phase, the microorganisms were diluted by a factor of 100 and then they were cultured for 3 h, again at 37 °C and 140 turns per minute. Afterwards, the bacteria were centrifuged (10 min, 20 °C, 4000 × *g*) and the supernatant was removed. The remaining pellet was diluted in PBS-buffer (Biochrome AG, Berlin, Germany), the optical density was adjusted in a photometer to 0.5 at a wavelength of 620 nm (Tecan, Salzburg, Austria) and the culture was stored again for 1 h at 37 °C and 140 turns per minute, and 96-well plates were from Greiner bio-one (Kremsmünster, Austria).

The chemical structures of the LPS from the mutants of *S. minnesota* are presented in Fig. 1b.

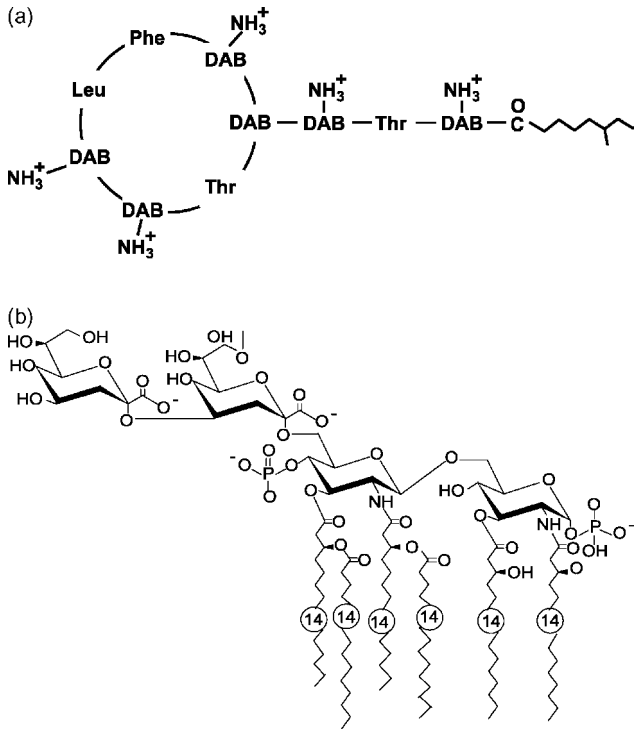

Fig. 1. (a) Chemical structure of polymyxin B (DAB: diamino butric acid) [21]. (b) Chemical structures of the LPS from *Escherichia coli* [18].

2.3. Determination of the minimal inhibitory concentration (MIC)

To measure the biological activity of a peptide and the sensitivity of a bacterial strain, it is necessary to quantify the concentration of the antimicrobial peptide needed to inhibit bacterial growth. For this purpose, 10 μl of a suspension of 10^6 bacteria/ml was diluted with 90 μl of PBS-buffer and treated with a dilution series of the antimicrobial peptide, starting with a concentration of 5 μmol/l and a dilution factor of 10. The bacterial samples were treated with the peptide and stored overnight at 37 °C in a 96-well plate (Greiner bio-one, Kremsmünster, Austria). The optical density was detected at a wavelength of 620 nm with a photometer. After subtraction of the optical density of the pure buffer, the optical density of the growth control (no peptide) was set to 100% growth. Therefore, the optical density is a measure for the bacterial concentration. The MIC was defined as the lowest concentration of peptide where the bacterial growth was significantly inhibited.

2.4. Isothermal titration calorimetry (ITC)

Microcalorimetric experiments of peptide binding to bacteria were performed on a MCS isothermal titration calorimeter (Microcal Inc., Northampton, MA, USA) at a temperature of 37 °C. The bacteria samples were filled into the microcalorimetric compartment (volume 1.5 ml) and the peptide at a concentration of 0.8 mM into the syringe compartment (volume 100 μl). The peptide solution was added 1800 s after thermal equilibrium has been reached into the cell, which was stirred

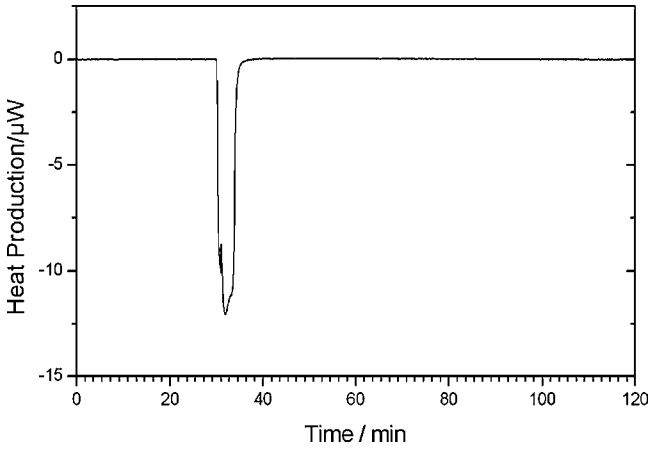

Fig. 2. Isothermal calorimetric titration of 0.8 mmol polymyxin B to a bacterial suspension with an optical density of 0.5, which corresponds to a bacterial concentration of 10^6 bacteria/ml.

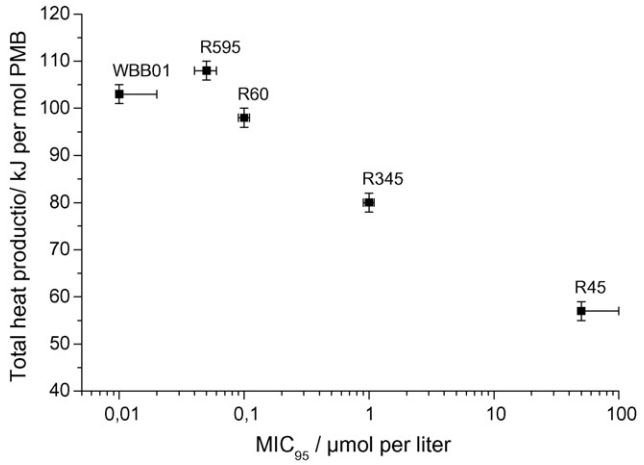

Fig. 3. Heat production of different bacteria. The heat production was plotted versus the minimum inhibitory concentration of the different bacterial strains (WBB01: *E. coli*; R595, R60, R345: *Salmonella minnesota*; R45: *Proteus mirabilis*).

constantly. After the measurements, a constant baseline was subtracted and the heat of interaction after the injection was plotted versus time. The total heat signal from the reaction was determined as the integrated power, i.e., the area under the peak. All titration measurements, performed at constant temperatures, were repeated at least two times. As control for the ITC experiments, PMB was titrated into pure buffer, and a small endothermic reaction due to dilution could be observed.

3. Results

The metabolic reactions under isothermal conditions of different PMB-resistant and -sensitive Gram-negative bacterial strains were measured at the physiological temperature of 37 °C. The bacteria were cultured under standard laboratory conditions (see above) and were washed in Phosphate-buffer to avoid any enthalpic effects of the fresh medium in which the antibiotic was solved and of metabolites of the bacteria. The storage of the bacteria in phosphate buffer also stopped their division in a way that their metabolism was reduced to a minimum. In Fig. 2, one typical experiment is shown indicating an exothermic reaction of the interaction of PMB with the bacterial cell envelope. As presented in Table 1, the molar enthalpies of the different bacterial strains show a clear dependence on the type of bacteria. The molar enthalpies differ from −108 kJ/mol (±1 kJ/mol) for PMB-sensitive *S. minnesota* R595 strain to 57 kJ/mol (±1 kJ/mol) for the resistant *P. mirabilis* R45. The other sensitive strains showed a molar enthalpy of 98 kJ/mol for *S. minnesota* R60, 80 kJ/mol for *S. minnesota* R345, and 103 kJ/mol for *E. coli* WBB01. It should be pointed out that in the case of the PMB-resistant strain R45 the produced heat of 57 kJ/mol was at least nearly a factor of two less than for the sensitive strains of *S. minnesota*.

Table 1
Molar enthalpies of different bacterial strains treated with polymyxin B at 37 °C

Bacterial strain	Enthalpy (kJ/mol)
Salmonella minnesota R595	−108 ± 1
Escherichia coli WBB01	−103 ± 1
S. minnesota R345	−80 ± 1
S. minnesota R60	−98 ± 1
Proteus mirabilis R45	−57 ± 1
Buffer (control)	+8

The final PMB concentration was 0.01 mM.

The amount of PMB needed to inhibit the growth of the different bacterial strains vary by nearly four orders of magnitude as expected, and the MIC varied from 0.01 μM for *E. coli* WBB01 strain to 50 μM for *P. mirabilis* R45 (Fig. 3).

4. Discussion

The increasing resistance of bacteria causes major problems in treating infections in human health care. This is the reason that research on this phenomenon increases constantly. This starts at an empirical level and proceeds to investigations of the detailed mechanisms on the molecular level, but calorimetric data are so far not available. Therefore, we studied the heat production of different bacterial strains in relation to their resistance to PMB.

The obtained results for PMB-induced killing of bacteria are in a range from 0.01 μg/ml for sensitive strains to 100 μg/ml for the resistant strain, which is in good agreement to values described in the literature [15–17]. Bacterial resistance against a specific antibiotic can have many different causes. The biochemical reactions in the cytoplasmic region can be completely changed by the absence of some key molecules, but the total heat production of the metabolic processes would only be slightly affected if these molecules are missing at the end of the metabolic pathway. Thus, the total heat production should mainly be dependent on the total amount of PMB incorporated into the membrane. The possibility of PMB-influx into the bacterium is mainly determined by the ability of the peptide to pass the outer membrane. Similar to other membrane-active peptides, this property is mainly influenced by the interaction with the LPS of the outer membrane. This interaction may be with the hydrophobic membrane anchor, lipid A, or the hydrophilic sugar

moiety. From our measurements, we can deduce that the influence of the sugar moiety of LPS is weaker than the dependence on the lipid A, when the thermal effects on R595 and R60 of *S. minnesota* are compared which differ by various monosaccharides (see Fig. 1b). A change in the chemical structure of the core region of LPS can also be responsible for the different sensitivity. This is in accordance what we see here, when the reactions of R595 (from *S. minnesota*), WBB01 (from *E. coli*) and R45 (from *P. mirabilis*) are compared. The main difference in these corresponding LPS is the net charge of the phosphates in the core oligosaccharide and the net charge of the lipid A backbone plus the two sugars [18]. This finding is not only reflected in the minimal inhibitory concentration, but also in the total heat production, which indicates that the amount of incorporated PMB into the cytoplasm is influenced by the chemical structure of the LPS within the outer membrane. From these data it may be deduced that the presence of the positively charged arabinose in the head group region of the LPS (linked to the first Kdo residue) reduces the negative charge density of LPS, and, probably more important, represents a steric hindrance for penetration of PMB into the outer membrane, explaining the reduced heat of interaction of PMB with the R45 bacteria.

It is known that the interaction of PMB with whole cells is temperature-dependent [19], which was interpreted as the change of membrane fluidity. The phase transition temperature of the outer membrane is higher than that of the cytoplasmic membrane [20], which is also an explanation for that the LPS of the bacterium is of high importance in understanding the different reactions to PMB.

We have investigated here the main calorimetric effects of PMB on binding to bacteria. As next step, a detailed analysis of the reactions under different physiological conditions as well as the temperature-dependence and the detailed characteristics of these reactions will follow.

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