Modulation of the Hydrophobic Domain of Polymyxin B Nonapeptide: Effect on Outer-Membrane Permeabilization and Lipopolysaccharide Neutralization

HAIM TSUBERY, ITZHAK OFEK, SOFIA COHEN, MIRIAM EISENSTEIN, and MATI FRIDKIN

Department of Organic Chemistry (H.T., M.F.) and Chemical Services Unit (M.E.), the Weizmann Institute of Science, Rehovot, Israel; and Department of Human Microbiology, Sackler Faculty of Medicine, Tel Aviv University, Tel-Aviv Israel (H.T., I.O., S.C.)

Received April 24, 2002; accepted August 05, 2002 This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Polymyxin B nonapeptide (PMBN), a cationic cyclic peptide derived from the antibacterial peptide polymyxin B, is capable of specifically increasing the permeability of the outer membrane (OM) of Gram-negative bacteria toward hydrophobic antibiotics. In this study, we evaluated the contribution of the hydrophobic segment of PMBN (i.e., D-Phe⁵-Leu⁶) to this activity. Accordingly, we synthesized four analogs of PMBN by replacing D-Phe⁵ with either with D-Trp or D-Tyr and Leu⁶ with Phe or Ala and evaluated their ability to bind cell-free lipopolysaccharide (LPS) and increase bacterial OM permeability. Compared with PMBN, [D-Tyr⁵]PMBN and [Ala⁶]PMBN possessed reduced LPS affinity (IC₅₀ = 2.5, 25, and 12 μM, respectively) and significantly reduced OM permeability and LPS neutralization activity. [Phe⁶]PMBN exhibited rather similar affinity to cell-free LPS (IC₅₀ = 5 μM) and the same OM permeability capacity as PMBN. However, [D-Trp⁵]PMBN, despite its similar affinity to cell-free LPS (IC₅₀ = 4 μM), had moderately reduced OM permeability capacity. These results demonstrate the significant role of the PMBN hydrophobic segment in promoting biological activity.

The bacterial endotoxin lipopolysaccharide (LPS) is the major antigen of the outer membrane (OM) of Gram-negative bacteria. The presence of LPS in circulation induces uncontrollable activation of immune cells followed by cytokine-mediated damage to blood vessels and a decrease in vascular resistance, frequently leading to collapse of organs and death (Karima et al., 1999). Hence, neutralization of the devastatin effects of LPS is a major target in combating endotoxicosis (Lynn and Cohen, 1995). LPS is composed of three major parts, one of which is lipid A, a highly conserved hydrophobic region. Lipid A is a phosphoglycolipid molecule composed of β-(1,6)-linked β-glucosamine disaccharide substituted by charged phosphate groups at positions 1 and 4 and 3-hydroxy saturated fatty acids of 12 to 16 carbon atoms (Zahringer et al., 1994). Lipid A, the toxic part of LPS, is a target for cationic proteins and peptides such as polymyxin B (Rietschel et al., 1987).

Polymyxin B (PMB) is a mixture of naturally occurring cationic cyclic decapeptide derivatives isolated from Bacillus polymyxa (Ainsworth et al., 1947; Benedict and Langlykke 1947; Stanly et al., 1947). PMB is highly bactericidal to Gram-negative bacteria and considered one of the most efficient cell-permeabilizing compounds (Evans et al., 1999). Largely because of its high-affinity binding to lipid A (Moore et al., 1986). Although PMB is bactericidal to multidrug-resistant Gram-negative bacteria and able to neutralize the toxic effects of released LPS, its therapeutic applications are very limited because of its relative high toxicity (Vinnicombe and Stamey, 1969; Kunin and Bugg, 1971). Because most of the toxic activity of PMB resides at the N-terminal fatty amino acid 6-methylheptanoic/octanoic-Dab, the removal of this segment by proteolytic cleavage, using ficin or papain, generated a nontoxic peptide named polymyxin B nonapeptide (PMBN) (Chihara et al., 1973, Duwe et al., 1986) (Fig. 1, Table 1). Although PMBN is an extremely poor antimicrobial compound, it is still capable, like PMB, of binding to LPS (Vaara and Viljanen, 1985) and preserving a significant OM-permeabilizing action, thus rendering Gram-negative bacteria susceptible to various hydrophobic antibiotics (Vaara and Vaara, 1983; Viljanen and Vaara, 1984). Such susceptibility was evidenced by a drastic sensitization of bacteria toward hydrophobic antibiotics such as rifampin, erythromycin, clindamycin, fusidic acid, and novobiocin against which the intact OM is an effective barrier (Vaara and Vaara, 1983). PMBN was able to protect mice challenged with Klebsiella pneumoniae in combination with erythromycin (Ofek et al.,

ABBREVIATIONS: LPS, lipopolysaccharide; OM, outer membrane; PMB, polymyxin B; PMBN, polymyxin B nonapeptide; Dab, 2,4-diaminobutyric acid; Cbz, benzoxycarbonyl; DMF, dimethylformamide; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; GRAVY, grand average of hydropathicity; ISB, Isotonic Sensitest Broth; CFU, colony forming unit; MIC, minimal inhibitory concentration; CD, circular dichroism; TNF, tumor necrosis factor; IL, interleukin; RP-HPLC, reversed phase–high-performance liquid chromatography; MM6, MONO-MAC-6.
1994). Structure-function analysis of PMBN has revealed the significance of the unique structural architecture of the naturally derived PMBN molecule (Tsbery et al., 2000a,b). The interaction of PMB as well as of PMBN with LPS may be mediated by two processes: electrostatic interactions between the positive charges of the peptidic Dab residues and the negative charges of the phosphates of LPS, and hydrophobic contacts of the N-terminal fatty acid 6-methylheptanoic/octanoic and D-Phe5-Leu6 of PMB with the hydrophobic core of the lipid A moiety of LPS (Srimal et al., 1996; Surajit et al., 1997; Pristovsek and Kidric, 1999). Several structural aspects pertaining to the contribution of the positive charges of PMBN to its interaction with LPS were recently reported (Tsbery et al., 2000a).

To better understand the contribution of the hydrophobic segment D-Phe5-Leu6 to the peptide-LPS association, the D-Phe5 residue was replaced either by D-Trp or D-Tyr and Leu6 by Phe or Ala. The interaction of the resultant PMBN analogs with LPS and their biological activities were investigated.

**Materials and Methods**

**Synthesis of PMBN Analogs.** All protected amino acids, coupling reagents, and polymers were obtained from Nova Biochemicals (Laufelfingen, Switzerland) and Bachem (Bubendorf, Switzerland). Synthesis-grade solvents were obtained from Labscan (Dublin, Ireland). Linear peptide chains were assembled by conventional solid phase synthesis, using an automated solid phase multiple peptide synthesizer (AMS-422; ABIMED, Langenfeld, Germany). 9-Fluorenylmethoxycarbonyl strategy was employed throughout the synthesis. Side-chain amino protecting groups for the C terminus (solution A) and 0.1% TFA in 75% acetonitrile in water (solution B). The column was eluted at t=40°C; 1:3, v/v) and centrifuged. The pellet was washed with the same mixture, dissolved in water/acetonitrile (2:3, v/v) and the solution was lyophilized. 

**Reversed-Phase HPLC Purification and Analyses.** The crude synthetic peptides were purified with a prepacked LichroCart RP-18 column (250 × 10 mm; 7-μm bead size; E. Merck, Darmstadt, Germany) employing a binary gradient formed from 0.1% TFA in water (solution A) and 0.1% TFA in 75% acetonitrile in water (solution B). The column was eluted at t = 6 min, B = 0%, and at t = 48 min, B = 60%, using a flow rate of 5 ml/min. For purity evaluation, analytical reversed-phase HPLC was performed using a prepacked Lichrospher-100 RP-18 column (250 × 4 mm, 5-μm bead size; E. Merck) (95% according to analytical HPLC). The final product was precipitated with cold tert-utylmethyl ether, centrifuged, washed with cold tert-utylmethyl ether, and lyophilized from water.

**TABLE 1**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence*</th>
<th>ESMS (m/z)</th>
<th>Amino Acid Analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMBN</td>
<td>TXcyclo[XFPLXXXT]</td>
<td>963.6 (962.6)</td>
<td>T, 1.92; X, 4.98; L, 1.0; F, 1.0</td>
</tr>
<tr>
<td>[Phe]PMBN</td>
<td>TXcyclo[XFXXXXXXT]</td>
<td>998.0 (997.1)</td>
<td>T, 1.99; X, 4.95; F, 2.0</td>
</tr>
<tr>
<td>[Ala]PMBN</td>
<td>TXcyclo[XFXXXXXXT]</td>
<td>921.9 (920.2)</td>
<td>T, 1.93; X, 5.09; A, 1.0; F, 1.0</td>
</tr>
<tr>
<td>[D-Trp]PMBN</td>
<td>TXcyclo[XWXLXXXT]</td>
<td>1002.6 (1001.4)</td>
<td>T, 1.78; X, 4.88; L, 1.0</td>
</tr>
<tr>
<td>[D-Tyr]PMBN</td>
<td>TXcyclo[XYLXXXT]</td>
<td>979.4 (978.6)</td>
<td>T, 1.71; X, 4.67; L, 1.0; Y, 1.0</td>
</tr>
</tbody>
</table>

* D-amino acid are in boldface. X = Dab.
* Trp destroyed upon hydrolysis procedure.

ESMS, electrospray ionization mass spectrometry.
with the following binary gradient: at t = 0 min, B = 10%, and at t = 40 min, B = 60% at a flow rate of 0.8 ml/min.

HPLC separations and analyses were performed using a liquid chromatography system (SP8800; Spectra-Physics, Stahnsdorf, Germany) equipped with a variable-wavelength absorbance detector (ABI 757; Applied Biosystems Foster City, CA). The column effluents were monitored by UV absorbance at 220 nm. Purity of peptides was \( > 98\% \) (yields, 30–45%). The corresponding fractions were collected, lyophilized, and analyzed after exhaustive acid hydrolysis and pre-column reaction with 6-aminooquinolyl-N-hydroxysuccinimidyl carbonate to ascertain amino acid composition (2690 separations Module; Waters, Milford, MA). Mass spectra analysis was performed to determine molecular weights (VG-platform-II electrospray single quadrupole mass spectrometer; Micro Mass, Manchester, UK).

PMBN. PMBN was prepared by proteolysis of PMB (Sigma, St. Louis, MO) with papain (Sigma) as described elsewhere (Danner et al., 1989). The crude product was purified (>98%) by HPLC, then analyzed and characterized as described above.

Dansyl-PMBN. Dansyl-PMBN was synthesized as described elsewhere (Tsbery et al., 2000a).

Calculation of Peptides Hydropathicity (GRAVY). Peptides grand average hydropathicity (GRAVY) was calculated using the Web-available program developed by Kyte and Doolittle (1982) (http://www.expasy.ch/cgi-bin/protscale.pl). To calculate relative hydropathicity values of the peptides, the Dab residues, (i.e., the unnatural amino acid) were replaced by Lys residues and the peptides were considered linear. Each amino acid was assigned a hydropathy index, a value reflecting its relative hydrophilicity and hydrophobicity. The sum of the hydropathy indices of a given sequence divided by the number of residues in the sequence generates the GRAVY score. In our case, the difference is in only one amino acid; hence, the GRAVY score reflects the different hydropathy indices of the residues at positions 5 and 6.

Molecular Modeling. The coordinates of PMB were obtained from Pristovsek and Kidric (1999). The model structure of LPS was constructed using the MSI package (MSI Inc., San Diego, CA). The complex PMB-lipid A was assembled in a way that the interactions were as described by Pristovsek and Kidric (1999). The model structure of the complex was energy minimized in vacuum. Initially, only the lipid A molecule was allowed to change, whereas the PMB molecule was fixed at the NMR structure. Subsequently, the restrictions were removed and both molecules were allowed to change. The complex PMBN-LPS was modeled by removal of residues -1 (6-methyl heptanoic acid) and 0 (Dab) from the above PMB-LPS complex and the energy was minimized again. The energy minimization was performed using the consistent-valence force field within the Discover module of the MSI package (MSI, Inc.). The convergence requirement was for the maximum derivative to be less than 0.001.

Determination of Minimal Inhibitory Concentration. The employed clinical isolates of Escherichia coli, K. pneumoniae, and Pseudomonas aeruginosa were obtained as described elsewhere (Oefe et al., 1994). The Gram-negative bacteria were grown on nutrient agar plates (Difco Laboratories, Detroit, MI) and kept at 4°C. Lyophilized aliquots of peptides (2 mg, determined by weight and ascertained by amino acid composition analysis) were dissolved in sterile double distilled water and filtered using a 0.2-μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI). An overnight culture in RPMI medium/10% newborn calf serum, 1 mM sodium pyruvate, 1% non-essential amino acids, and 9 μg/ml insulin) in polypropylene tubes. MONO-MAC-6 (MM6) cells (5 x 10^6) were added to a final concentration of 50 μg/ml, and both molecules were allowed to change. The structure was evaluated using CD measurements. [d-Trp]PMBN and [d-Ala]PMBN were equally hydrophobic to PMBN whereas [d-Tyr]PMBN and [d-Leu]PMBN were less hydrophobic (Table 4). The hydropathicity scale (Kyte and Doolittle, 1982), however, indicated that [d-Trp]PMBN was much less hydrophobic than PMBN (Table 4).

Inhibition of Cytokine Release. Peptide solutions (1, 10, and 100 μM, final concentration) were incubated (10 min, 37°C) with E. coli-LPS (20 ng/ml, final concentration) in an assay medium (RPMI medium/10% newborn calf serum, 1 mM sodium pyruvate, 1% non-essential amino acids, and 9 μg/ml insulin) in polypropylene tubes. MONO-MAC-6 (MM6) cells (5 x 10^6) were added to a final concentration of 50 μg/ml, and both molecules were allowed to change. The structure was evaluated using CD measurements. [d-Trp]PMBN and [d-Ala]PMBN were equally hydrophobic to PMBN whereas [d-Tyr]PMBN and [d-Leu]PMBN were less hydrophobic (Table 4).

The peptides' structure was evaluated using CD measurements. [d-Trp]PMBN and [d-Ala]PMBN (0.2 mM) in phosphate buffer exhibited a random structure similar to 0.2 mM PMBN. A minor difference in the CD pattern was observed at 218 to 230 nm. [d-Trp]PMBN at the same concentration (0.2 mM) exhibited a maximal negative ellipticity at 200 nm and an additional maximal negative ellipticity at 220 nm. [d-Ala]PMBN exhibited reduced ellipticity compared with PMBN and a maximal negative ellipticity at 297 nm (Fig. 2).

Antimicrobial and OM Permeabilization Activities of PMBN Analogs. Unlike PMBN, none of the analogs was active against P. aeruginosa (MIC >250 μg/ml, Table 2). The peptides' (50 μg/ml) potency to increase the bacterial OM permeability toward novobiocin was evaluated. (Phe6)PMBN was added to a quartz cuvette containing LPS solution (2 ml, 3 μg/ml, –2 x 10^-7 M) in 5 mM HEPES, pH 7.2, and allowed to equilibrate at room temperature for 10 to 15 min. Subsequently, small portions (5–10 μl) of peptide solutions (1 x 10^-8–1 x 10^-5 M) were added. Inhibition of fluorescence was measured 5 min after each addition of peptides. Percent inhibition was plotted as a function of the added peptide concentration and IC50 values were calculated from maximal specific displacement (I_max).

Circular Dichroism (CD) Studies. CD spectra were recorded on an Aviv-202 circular dichroism spectrometer (Lakewood, NJ). Duplication scans over a wavelength range of 190 to 250 nm were taken at a chart speed of 12 nm/min in a 0.1-cm path-length quartz cell at room temperature. Peptides were dissolved in 5 mM phosphate buffer, pH 7.2, at a final concentration of 0.2 mM. A baseline was recorded and subtracted after each spectrum. Ellipticity is reported as the mean residue ellipticity [θ] in degrees cm^2 dmol^-1 x 10^-3.
was as potent as PMBN, whereas [Ala<sup>6</sup>]PMBN was 4- to 8-fold less active than PMBN (Table 3). [D-Trp<sup>5</sup>]PMBN was 8-fold less potent in the OM permeabilization assay compared with PMBN. The activity of [D-Tyr<sup>5</sup>]PMBN, however, was very weak (Table 3).

**LPS Binding.** The interaction of the PMBN analogs with the bacterial cell-free LPS was quantified using the dansyl-PMBN displacement assay. Table 4 shows that the peptide concentrations required for 50% displacement were 4 and 5 μM for [D-Trp<sup>5</sup>]PMBN and [Phe<sup>6</sup>]PMBN, respectively, similar to PMBN (IC<sub>50</sub> = 2.5 μM). However, [D-Tyr<sup>5</sup>]PMBN and [Ala<sup>6</sup>]PMBN exhibited significantly lower potency compared with PMBN (Table 4).

**Neutralization of LPS Toxic Effects.** The ability of PMBN and its analogs to neutralize stimulatory effects of LPS on the human monocyte cell line MM6 was tested. Peptides were preincubated with E. coli- LPS and the mixture was allowed to interact with the MM6 cells. Levels of released TNFα and IL-6 were measured after 4 and 18 h, respectively, using enzyme-linked immunosorbent assay. As shown in Fig. 3, stimulation of MM6 with LPS (20 ng/ml) triggered the release of ~40 and ~70 ng/ml of IL-6 and TNFα, respectively. PMBN and [D-Trp<sup>5</sup>]PMBN were equally potent inhibitors of TNFα release (95–23%). A similar effect was observed for the inhibition of IL-6 release. PMBN and [D-Trp<sup>5</sup>]PMBN inhibited IL-6 release at the range of 75–20% in a dose-dependent manner (1–100 μM). However, [D-Tyr<sup>5</sup>]PMBN was a much weaker inhibitor, causing no significant inhibition either in the TNFα or the IL-6 assay (20 and 10%, respectively). As shown, PMB exhibited maximal inhibition capacity even at 1 μM concentration.

**Molecular Modeling of Peptide-LPS Complex.** The structure of PMB bound to LPS was determined by NMR spectroscopy (Pristovsek and Kidric, 1999). Based on the coordinates provided for PMB, a model corresponding to 1:1 PMB/lipid A complex was generated (Fig. 4). The complex is characterized by electrostatic interactions between four of the five positive side chains of PMB (positions 0, 4, 7, and 8, Fig. 4) and two of the negative phosphate groups of the phosphorylated lipid A head-groups. The hydrophobic side chains at the N terminus and at positions 5 and 6 of PMB interact with the aliphatic chains of lipid A. The PMBN-LPS complex was generated upon removal of residues –1 (6-methyl heptanoic acid) and 0 (Dab) from the PMB molecule in the PMB-LPS complex and energy re minimization. The minimized structure suggests that the interaction between the positive Dab moiety at position 0 and the phosphate group in lipid A is replaced by an electrostatic interaction between Dab<sup>2</sup> of PMBN (numbering as for PMB) and the same phosphate group. The positive side chain of Dab<sup>2</sup> in PMB is exposed to the solvent in the PMB-LPS complex (Fig. 4). The hydrophobic segment n-Phe<sup>5</sup>-Leu<sup>6</sup> still interacts with the aliphatic chains of lipid A.

**TABLE 3**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>E. coli</th>
<th>K. pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novobiocin</td>
<td>62</td>
<td>250</td>
</tr>
<tr>
<td>Novobiocin + PMBN</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Novobiocin + [Phe&lt;sup&gt;6&lt;/sup&gt;]PMBN</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Novobiocin + [Ala&lt;sup&gt;6&lt;/sup&gt;]PMBN</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Novobiocin + [n-Trp&lt;sup&gt;5&lt;/sup&gt;]PMBN</td>
<td>8</td>
<td>31</td>
</tr>
<tr>
<td>Novobiocin + [n-Tyr&lt;sup&gt;5&lt;/sup&gt;]PMBN</td>
<td>31</td>
<td>125</td>
</tr>
</tbody>
</table>

**TABLE 4**

Peptide affinity to LPS and relative hydrophobicity

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; μM</th>
<th>t&lt;sub&gt;50&lt;/sub&gt; min</th>
<th>Hydropathicity (GRAVY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMBN</td>
<td>2.5</td>
<td>21.3</td>
<td>−1.589</td>
</tr>
<tr>
<td>[Phe&lt;sup&gt;6&lt;/sup&gt;]PMBN</td>
<td>5</td>
<td>21.3</td>
<td>−1.700</td>
</tr>
<tr>
<td>[Ala&lt;sup&gt;6&lt;/sup&gt;]PMBN</td>
<td>12</td>
<td>15.7</td>
<td>−1.811</td>
</tr>
<tr>
<td>[n-Trp&lt;sup&gt;5&lt;/sup&gt;]PMBN</td>
<td>4</td>
<td>21.7</td>
<td>−2.000</td>
</tr>
<tr>
<td>[n-Tyr&lt;sup&gt;5&lt;/sup&gt;]PMBN</td>
<td>25</td>
<td>16.4</td>
<td>−2.044</td>
</tr>
</tbody>
</table>

Fig. 2. Circular dichroism spectra of PMBN and its two analogs. Measurements were taken with peptide solutions (0.2 mM) in phosphate buffer (5 mM, pH 7.5) at 25°C. (α) is the mean residue ellipticity × 10<sup>3</sup> in degrees cm<sup>2</sup> dmol<sup>−1</sup>.
Discussion

The interaction of PMB and PMBN with their bacterial target molecule, LPS, was extensively studied in recent years. The intermolecular peptide-lipid associations were attributed to the amphiphilic features of PMB, involving primarily the hydrophobic face of the peptide enforced into an appropriate alignment by the positively charged side chains of the 2,4-diaminobutyric acid residues (Srimal et al., 1996). Similar structural considerations were deduced and elaborated from two-dimensional NMR and molecular dynamics studies of PMBN and E. coli-LPS (Bhattacharjya et al., 1997; Pristovsek and Kidric, 1999). According to the latter study, PMB and PMBN share a type II β-turn structure for the free peptide and an envelope-like fold of the peptide ring for the LPS-bound peptide. The present structure-function study of PMBN focused on the d-Phe5-Leu6 segment, the peptide ring hydrophobic domain of PMBN. As shown in our previous study, the substitution of d-Phe5 with l-Phe (i.e., construc-

![Graph showing inhibition of TNFα and IL-6 release](image_url)

Fig. 3. Inhibition of TNFα (A) and IL-6 (B) release from LPS-stimulated MM6 cells. Peptides (1–100 μM) were incubated (10 min) with LPS (20 ng/ml) and MM6 cells (0.5 × 10⁶) were added. Levels of TNFα and IL-6 were detected after 4 and 18 h, respectively. Data are expressed as the percentage from baseline (20 ng/ml LPS, normalized to 100%) and represented as mean ± S.D. derived from three experiments.
tion of [L-Phe5]PMBN resulted in loss of activity (Tsubery et al., 2000a). This finding is in line with the notion that position 2 of all naturally occurring polymyxins is occupied by the D-form of Trp (Atkinson and Pelton, 1992). The CD spectra of [Ala6]PMBN was identical to that of PMBN, whereas position 6 is occupied by the L-form of either Leu, Ile, or Thr. In the present study, PMBN was modified so that the configuration at positions 5 and 6 was retained but the hydrophobicity was changed.

According to the hydrophobicity scale the amino acids follow the order Phe > Leu > Trp > Ala > Tyr (Kyte and Doolittle, 1982; Engelman et al., 1986). The hydrophobicity of the various newly synthesized PMBN analogs was evaluated experimentally using RP-HPLC as well as calculated based on the grand average of hydropathicity (GRAVY) program developed by Kyte and Doolittle (1982). According to RP-HPLC, the peptides hydrophobicity follow the order [D-Trp5]PMBN > PMBN > [Phe6]PMBN > [Ala6]PMBN > [n-Tyr5]PMBN. However, the GRAVY scale generated the following order: PMBN > [Phe6]PMBN > [Ala6]PMBN > [D-Trp5]PMBN > [n-Tyr5]PMBN (Table 4). The difference might perhaps be caused by the actual overall structures that the peptides assume in the aqueous-organic milieu (acetoni-water).

The present CD measurement show that [n-Trp5]PMBN has a relative minor negative shoulder at 218 to 240 nm compared with PMBN and exhibits a rather marked negative band centered at 222 nm. This additional maximal negative ellipticity, however, could be attributed to the contribution of the indole moiety of Trp (Atkinson and Pelton, 1992). The CD spectra of [Ala6]PMBN was identical to that of PMBN, whereas [Phe6]PMBN exhibited a CD pattern with reduced ellipticity. However, no major peptide structural changes were found among the four peptides and all displayed random coil structure.

Although most bacteria are resistant to the bactericidal activity of PMBN, P. aeruginosa is an exception. None of the newly synthesized PMBN analogs was able to inhibit the growth of P. aeruginosa. So far, any modification made in PMBN resulted in loss of direct bactericidal activity toward P. aeruginosa (Tsubery et al., 2000a,b). When the peptides were evaluated for their ability to permeate the bacterial OM, [n-Tyr5]PMBN was found to be inactive, whereas [D-Trp5]PMBN and [Ala6]PMBN exhibited reduced OM permeabilizing activity compared with PMBN. [Phe6]PMBN, however, was as active as PMBN. This loss and reduced activities of the respective peptides might perhaps be explained by their GRAVY (Kyte and Doolittle, 1982) (that is, the lower the hydropathicity, the lower the antibacterial activity). Indeed, [Phe6]PMBN has an OM permeabilizing ability identical to that of PMBN and the values of their hydropathicity index are close. These observations are consistent with the finding that, as with other antimicrobial peptides, amphipathicity is an essential parameter for the activity of PMB and its analogs (Liao et al., 1995; Srimal et al., 1996; Bhattacharjya et al., 1997; Hancock, 1997; Pristovsek and Kidric, 1999).

However, the affinity of the peptides to cell-free LPS as evaluated by the Dansyl-PMBN displacement assay had greater correlation with the hydrophobicity scale drawn by RP-HPLC (Table 4). Thus, the interaction of the peptides with cell-free LPS is somehow different from their interaction with cell-bound LPS.

The interaction of LPS with immune cells via its receptor (CD 14) results in stimulatory effects leading, among others, to enhanced release of cytokines such as TNFα and IL-6 (Viriyakosol and Kirkland, 1995). The ability of PMBN and its analogs [n-Trp5]PMBN and [n-Tyr5]PMBN to neutralize the stimulatory effect of LPS was evaluated. This capacity relates to the binding of the peptides to the LPS molecule and impairing its association with cognate immune cell receptors. Thus, both PMBN and [n-Trp5]PMBN bound to LPS and prevented its interaction with the LPS-receptor on MM6 cells in a similar dose-dependent manner. [n-Tyr5]PMBN, however, was not able to inhibit the release of TNFα and IL-6. These results are in good correlation with the affinity of the peptides to cell-free LPS and to the hydrophobic scale drawn by RP-HPLC.

The structures of PMB and PMBN and its analogs bound to LPS were modeled based on coordinates provided for PMB (Pristovsek and Kidric, 1999) (Fig. 4). Elimination of the positively charged residue Dab0, which interacts with the negatively charged phosphate group in the PMB-LPS complex, is not likely to significantly weaken the LPS-peptide interaction. This is partly because of the conformational change of the side chain at position 2, allowing for the efficient interaction of the Dab0 residue with the negatively charged phosphate group and partly because the exposed phosphate can interact with water (Fig. 4). Thus, modifications at position 2 may lead to the weakening of the interaction. This assumption is supported by our previous observation that substitution at position 2 with Lys reduces the peptide OM permeabilizing activity as well as its affinity to LPS (Tsubery et al., 2000a). Preservation of the cationic nature of the peptides is of great significance in relation to their ability to enhance penetration through biological membrane. However, it was shown that various linear cationic analogs of PMBN analogs are devoid of activity, suggesting that the cyclic structure is of major significance (Vaara, 1991).

The hydrophobic interaction of PMBN with the bacterial
membrane is of great significance for its OM permeabilization activity. Indeed, the parent PMB molecule has two hydrophobic regions, the fatty acid moiety at the N terminus and the β-Pheβ-Leuβ segment in the peptide ring. The removal of the fatty tail abolished its direct antimicrobial activity. Modulation of the hydrophobic segment of PMBN reduced its OM permeabilization activity. The proximity between the aromatic ring of β-Pheβ and the side chain of Leuβ promotes the formation of the β-turn in the peptide (Pristovsek and Kidric, 1999). Thus, substitution at these positions in PMBN with less hydrophobic residues may impair the stability of this β-turn. Such a structural change may affect the amphiphilic nature of the peptide and, in turn, weaken its LPS binding and activity.

References


