Polymyxin B nonapeptide (PMN) is a derivative of polymyxin B, an α,γ-diaminobutyric acid-rich decapetide from Bacillus polymyxa that displays antimicrobial and lipopolysaccharide (LPS)-antagonistic activities. The conformations of PMN in aqueous solution as well as in the LPS-bound state have been studied by 1H two-dimensional nmr methods in conjunction with molecular dynamics techniques. The aqueous structure of the free peptide is characterized by a type I' β-turn centered around D-Phe and Leu, and an inverse β-turn at Thr. The LPS-bound conformation of PMN was studied by transferred nuclear Overhauser effect experiments. The essential features of the cyclic portion of free aqueous PMN are mostly preserved when the peptide is bound to LPS; however, the linear dipeptide fragment as well as the side chains of the heptapeptide ring show a conformational change and a reduction in mobility. The LPS-bound PMN structure was used to construct a model of the lipid A-polymyxin B (decapetide) complex, which allows the rationalization of several experimental observations concerning the binding of polymyxin to, and consequent neutralization of, the toxicity of LPS, and may be of value in the rational therapeutic targeting of endotoxin. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

Lipopolysaccharides (LPS), structural components of gram-negative bacterial outer membranes, elicit a wide range of toxic effects in a variety of organisms, with humans among the most susceptible species. The release of LPS into systemic circulation, which occurs commonly as a consequence of major gram-negative bacterial infections, leads to a constellation of symptoms
termed "endotoxic shock," characterized by hemodynamic abnormalities, coagulopathy, and multiple system organ failure. No therapeutic modality of documented value is available at the present time and mortality due to this syndrome has remained essentially unchanged over the past decade at around 60%. Our understanding of the structural aspects of LPS and of the cellular mechanisms underlying host responses to this toxin has now advanced to such a level as to render the rational therapy of endotoxic-related disease states a tractable goal. Indeed, several experimental approaches, targeting almost every mechanism and process known or thought to be operational in endotoxicity, are being evaluated for possible clinical benefits.

The structurally highly conserved amphiphilic lipid moiety of LPS, termed "lipid A" has been shown, by total organic synthesis, to represent the toxic center of endotoxin. A possible therapeutic option, therefore, is the sequestration, or removal, of LPS, which can be accomplished with agents that bind lipid A, preventing its recognition by endotoxin-specific receptor molecules. Polymyxin B (PMB), a cationic amphiphilic decapetide antibiotic obtained from Bacillus polymyxa, binds to the lipid A portion of whole LPS with an apparent dissociation constant of 0.4 μM and the resultant complex is virtually devoid of toxicity. Polymyxin B, however, is toxic, which, while proscribing its parenteral use, has stimulated the search for nontoxic polymyxin analogues as candidate LPS antagonists. An understanding of the structural aspects of polymyxin and its complexes with lipid A would serve as a useful paradigm for the rational development of lipid A binding agents. The crystal structure of polymyxin B has not been reported and few studies exist in the literature on the solution conformation of the peptide, none of which are of sufficient detail to permit considerations of the interaction of the antibiotic with LPS.

This paper describes the conformational characterization of polymyxin B nonapeptide (PMN) in water in its free and LPS-bound state by two-dimensional (2D) nmr and molecular dynamics procedures. The nonapeptide derivative lacks a single N-methyloctanoyl-diaminobutyric acid residue of the parent polymyxin B molecule (Figure 1). We elected to characterize the nonapeptide since polymyxin B decapetide aggregates in solution with consequent loss of spectral resolution. Furthermore, the decapetide, but not the nonapeptide, precipitates in the presence of such concentrations of LPS as are necessary for nmr experiments. The LPS-polymyxin B complex, modeled using the LPS-bound conformation of PMN as a template, permits the rationalization of several experimental observations concerning the binding of the polymyxins, and subsequent neutralization of the activity of lipid A and LPS, and may be of value in the design of endotoxic antagonists.

FIGURE 1 The primary structure of PMN with the numbering scheme of the amino acids shown. The Polymyxin B decapetide has an additional N-3-methyloctanoyldiaminobutyric acid shown in the box.

MATERIALS AND METHODS

PMN was obtained from Boehringer Mannheim as the dihydrochloride salt. The purity of PMN was found to be about 90% by high performance liquid chromatography on a reverse-phase C18 column using a methanol/water binary gradient with 0.1% trifluoroacetic acid (methanol: 40-90%; retention time: 11.82 min). Seven milligrams of the peptide were dissolved in 0.5 mL of 90% H2O/10% D2O (ca. 3.5 mM). All experiments were performed at pH 3.0 (adjusted with HCl) since at acidic pH, all the Dab γ-amino groups are protonated, which helped alleviate the potential problem of peptide aggregation. LPS from Escherichia coli 0111:B4 and D2O (99.9%) were purchased from Sigma Chemicals, Inc. Since the polysaccharide moiety of LPS is considerably heterogeneous, concentrations of LPS are given as mass per unit volume.

NMR Experiments

1H-nmr spectra were acquired on a Bruker AMX-400 spectrometer (400 MHz) equipped with an Aspect 3000 computer. A low power irradiation (55 dB) of the water signal was applied during the relaxation delay to suppress the solvent resonance. Double quantum-filtered correlated spectroscopy (DQF-COSY) and total COSY (TOCSY) experiments were performed in the phase-sensitive mode to assign the spin systems. The mixing time in the TOCSY experiments was 60 ms. TOCSY and DQF-COSY spectra were acquired with a maximum of 512 experiments of 40 scans each. Spectral width...
(4504 Hz) was always identical in both dimensions with the time domain in $t_2$ set to 1 K data points.

Two-dimensional nuclear Overhauser effect spectroscopy (NOESY) and rotating frame NOESY (ROESY) spectra were acquired in the pure absorption mode using time-proportional phase increments along $t_1$. NOESY and ROESY spectra were collected with mixing times of 300 and 400 ms respectively, with 512 experiments of 72–88 scans each. For all the experiments the recycle delay was set to 1.5–2 s. All 2D data were zero filled to 1024 points in both dimensions and multiplied by a $\pi/2$-shifted square sine-bell function prior to Fourier transformation. The probe temperature was maintained at 298 K. In temperature-dependence experiments performed to obtain the temperature coefficients of the amide protons, sets of one-dimensional (1D) experiments were performed at various temperatures.

In order to examine the binding of PMN to LPS, line-broadening experiments were performed by successively adding small aliquots of a concentrated stock solution of LPS (20 mg/mL in $D_2$O) to PMN (3.5 mM; in 90% H$_2$O/10% $D_2$O). Two-dimensional transferred NOE (TRNOE) experiments were carried out using a mixture of PMN and LPS that yielded moderately broadened PMN resonance. This mixture corresponds to a 20:1 w/w ratio of PMN (3.5 mM) and LPS (the considerable heterogeneity of the polysaccharide portion of LPS precludes precise concentration estimates of LPS). A mixing time of 200 ms at 298 K.

**Computational Methods**

A crude molecular model of PMN was built using INSIGHT-II (BIOSYM Technologies, CA) on a Silicon Graphics Iris workstation. The dianinobutyric acid residue was constructed from lysine; all other residues were available in the INSIGHT amino acid library. Cyclization of the cyclic portion of the peptide was achieved by creating a peptide bond between the $\gamma$-NH of Dab and the carboxyl group of Thr. All energy minimization procedures were performed using the DISCOVER module in INSIGHT. The Consistent Valence Force Field was used with Morse harmonic potential energy terms for bond lengths, and bond and dihedral angles, with cross terms for charge-charge interactions. A dielectric constant of 1.0 was used. The $\gamma$-amino functions of the Dab residues were not protonated to avoid distortions due to charge repulsive effects that are to be expected in the absence of adequate solvent screening. A few cycles of steepest descent minimization relaxed short contacts; this was followed by minimization using the conjugate gradients algorithm with the convergence tolerance set to 0.001 of the average absolute energy derivative.

Interresidue NOE-derived constraints, as well as H-bond constraints derived from the temperature coefficients, were considered in generating acceptable models. Since all the interresidue NOEs were more or less equally intense, the upper limit of the NOE distance cutoff was empirically set to 3 Å, a reasonable approximation for relatively small molecules. Main-chain to side-chain NOEs were few and very weak, and therefore were not considered as constraints. For H bonds, the lower limit of the $N \leftrightarrow O$ distance was 2.5 Å. A force constant of 50 kcal/mol was applied on all distance constraints as a penalty function for violation of distance criteria. All generic distance constraints are shown in Table I.

Molecular dynamics (MD) simulations were performed in an effort to search for alternate conformations that are consistent with the experimentally observed distance-geometry constraints and to examine the conformational flexibility of the molecule. MD trajectories were computed at 300 K using the crude starting model without constraints employing the Verlet Leapfrog algorithm with the same energy terms as those used in the energy minimization procedures. The molecule was allowed to equilibrate at 300 K in vacuo and in the absence of solvent molecules until the temperature drifts stabilized completely (usually less than 1 ps). Trajectories were then obtained for 10 ps with an integration time step of 2 fs; atomic coordinates were recorded every 1 ps, resulting in an ensemble of 10 structures. The distance constraints were then imposed on these structures and were energy minimized to complete convergence. Alternatively, trajectories were also computed with NOE and H-bond distance constraints imposed. The former procedure of unconstrained dynamics simulation followed by restrained energy minimization was preferred to the latter since this method resulted in a greater exploration of conformational space as judged by inspecting dihedral trajectory plots, and all MD results discussed in this paper are those derived from the former procedure.

Distance constraints derived from TRNOE experiments were used to model the LPS-bound conformation of PMN. In these procedures, all observed NOEs (within the backbone protons, backbone to side-chain and side-chain-sidechain protons)
were used as constraints in molecular modeling performed as described above. The PMB decapptide was then constructed from the LPS-bound PMN structure by adding a N-3-methyloctanoyl-Dab residue to Dab. This was then docked onto a molecular model of lipid A. The docking was performed interactively such that electrostatic interactions between the lipid A phosphates and the \( \gamma \)-amino groups of the Dab residues were maximal in view of the primarily electrostatic mode of interaction of polymyxin with lipid A as well as other anionic phospholipids (see Discussion). The resultant complex was then subjected to a few hundred cycles of steepest descent energy minimization to relieve short contacts.

### RESULTS

**Assignments of Amino Acid Spin Systems**

Assignment of the proton resonances were done using 2D-TOCSY, DQF-COSY, ROESY, and NOESY experiments. All spin systems could be assigned unambiguously using TOCSY and COSY spectra in water. The resonances of D-Phe, Leu, and Thr were assigned by their characteristic chemical shifts of the \( C^\alpha \), \( C^\beta \), and \( C^\gamma \), respectively (Table 1): the large downfield chemical shifts of the \( C^\alpha \) protons of the Thr residues helped assigning both of them. The NH resonances of the

<table>
<thead>
<tr>
<th>Residue</th>
<th>NH</th>
<th>( C^\alpha )</th>
<th>( C^\beta )</th>
<th>( C^\gamma )</th>
<th>Others</th>
<th>( \Delta h/dT \times 10^{-3} ) (ppm-(K^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr(^a)</td>
<td>—</td>
<td>4.01</td>
<td>4.25</td>
<td>3.32</td>
<td></td>
<td>4.6</td>
</tr>
<tr>
<td>Dab(^a)</td>
<td>8.99</td>
<td>4.57</td>
<td>2.15, 2.25</td>
<td>3.2</td>
<td>NH: 7.74</td>
<td>4.4; NH: 4.0</td>
</tr>
<tr>
<td>Dab(^b)</td>
<td>8.61</td>
<td>4.34</td>
<td>1.90; 1.98</td>
<td>3.13</td>
<td></td>
<td>7.9</td>
</tr>
<tr>
<td>Dab(^b)</td>
<td>8.50</td>
<td>4.35</td>
<td>2.01; 2.06</td>
<td>3.13</td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>D-Phe(^a)</td>
<td>8.75</td>
<td>4.60</td>
<td>3.10, 3.09</td>
<td>0.85</td>
<td>0.70; 0.75</td>
<td>6.6</td>
</tr>
<tr>
<td>Leu(^a)</td>
<td>8.58</td>
<td>4.22</td>
<td>1.31; 1.42</td>
<td>1.22</td>
<td>Ring: 1H: 7.2; 7.4</td>
<td>6.2</td>
</tr>
<tr>
<td>Dab(^b)</td>
<td>8.23</td>
<td>4.32</td>
<td>2.28</td>
<td>3.13</td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>Dab(^b)</td>
<td>8.70</td>
<td>4.29</td>
<td>2.24</td>
<td>3.13</td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>Thr(^a)</td>
<td>7.91</td>
<td>4.21</td>
<td>4.28</td>
<td>1.22</td>
<td></td>
<td>4.2</td>
</tr>
</tbody>
</table>
five Dab residues were fairly well dispersed in 1D spectra and their spin systems were clearly distinguished in the TOCSY spectra (Figure 2); sequence-specific assignments for the Dab residues were performed on the basis of the observed NOEs to Phe, Leu, Thr, and Thr resonances in ROESY spectra (Figure 3a and b). The amino terminus Thr CαH appears at high field (4.01 ppm) and does not yield any NH/CαH cross peak in the TOCSY (Figure 2) or COSY spectra (not shown) enabling the assignment of the Dab spin system due to strong NOEs from Thr CαH and CβH protons (Figure 3a). Dab was assigned from sequential NOEs from Dab and Dab CαH, and NH, respectively. The side-chain NαH group of Dab that is bonded with the carbonyl of Thr could be distinguished from its characteristic NOEs with Thr CαH and CαH resonances. Dab, Dab, and Dab were assigned by the sequential NOEs from Phe, Leu, and Thr residues. The TOCSY spec-
tra (Figure 2) also reveal conformation-dependent chemical shift perturbations of Leu<sup>6</sup>, Dab<sup>4</sup>, and Dab<sup>4</sup> side-chain protons. The two nonequivalent methyl resonances of Leu<sup>6</sup> are moved upfield (0.2 ppm) from the position reported for unstructured polypeptides, presumably a result of ring current effects due to the proximal D-Phe<sup>3</sup> residue. Likewise, the chemical nonequivalence of the C<sup>α</sup>H<sub>3</sub>S and C<sup>α</sup>H<sub>3</sub>S resonances of Dab<sup>4</sup> are also found at relatively high field positions. The chemical shifts perturbations may be indicative of specific side-chain interactions involving the residues D-Phe<sup>3</sup> and Leu<sup>6</sup> and Dab<sup>4</sup>, which are not conformationally averaged. The C<sup>α</sup>H<sub>3</sub>S protons of Dab<sup>4</sup> also nondegenerate, appear at the highest field among the Dab residues, probably a consequence of the incorporation of its side chain into the cyclic backbone. The vicinal proton coupling constants (J<sub>Hα-NH</sub>) for aqueous PMN obtained

**FIGURE 3** (a) Partial 400 MHz ROESY spectrum of PMN in 90% H<sub>2</sub>O/10% D<sub>2</sub>O at 298 K, pH 3.0 (400 ms mixing time). Sequential C<sup>α</sup>H<sub>3</sub>/NH intrar residues, and D-Phe<sup>3</sup> ring protons to Leu<sup>6</sup> 4-methyl protons NOEs are indicated. X denotes α,γ-diaminobutyric acid (Dab). (b) Partial 400 MHz ROESY spectrum of PMN in 90% H<sub>2</sub>O/10% D<sub>2</sub>O at 298 K, pH 3.0 (400 ms mixing time), showing the NH/NH cross peak between Leu<sup>6</sup> and Dab<sup>4</sup>.
from 1D spectra which provide dihedral angle information [47] are listed in Table III.

Temperature Dependence

Temperature dependence of the chemical shifts of the amide protons were measured in order to identify the NH protons involved in intramolecular hydrogen bonding. The temperature coefficient of Dab7 NH is very low (0.85 ppm K⁻¹) indicative of its buried nature. The N'7H of Dab6 (4.0 ppm K⁻¹) also has a relatively low temperature coefficient in water, suggestive of a moderate degree of solvent shielding (Table III). It is noteworthy that in an early study of polymyxin B, two unassigned NH resonances were shown to be solvent shielded. All other NH resonances had high temperature coefficients characteristic of solvent-exposed protons.

Analysis of ROESY Spectra of PMN in Water

In NOESY spectra of aqueous PMN, NOE cross peaks for the protons of the cyclic portion do not appear, whereas NOEs were observed between
Table III  $^{3}J_{\text{HNCCH}}$ Coupling Constants and Corresponding Dihedral Angles for PMN in Water

<table>
<thead>
<tr>
<th>Residue</th>
<th>$^{3}J_{\text{HNCCH}}$ (Hz)</th>
<th>$\phi_{c}$</th>
<th>$\phi_{\text{mean}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr$^1$</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dab$^2$</td>
<td>7.0</td>
<td>+60; -78; -155</td>
<td>-82.6 ± 8.3</td>
</tr>
<tr>
<td>Dab$^3$</td>
<td>7.9</td>
<td>-95; -150</td>
<td>-120.6 ± 24.4</td>
</tr>
<tr>
<td>Dab$^4$</td>
<td>6.7</td>
<td>+40; +70; -80; -160</td>
<td>-101.6 ± 23.8</td>
</tr>
<tr>
<td>D-Phe$^5$</td>
<td>5.1</td>
<td>+20; +100; -75; -175</td>
<td>67.4 ± 4.8</td>
</tr>
<tr>
<td>Leu$^6$</td>
<td>7.6</td>
<td>-85; -150</td>
<td>-95.0 ± 12.2</td>
</tr>
<tr>
<td>Dab$^7$</td>
<td>5.5</td>
<td>+30; +82; -70; -170</td>
<td>-102.4 ± 24.5</td>
</tr>
<tr>
<td>Dab$^8$</td>
<td>4.9</td>
<td>+25; -65; +50; -170</td>
<td>-70.8 ± 4.5</td>
</tr>
<tr>
<td>Thr$^9$</td>
<td>7.6</td>
<td>-95; -160</td>
<td>-76.4 ± 4.0</td>
</tr>
</tbody>
</table>

* The $\phi$ values obtained using the expression $^{3}J_{\text{HNCCH}} = 8.4 \cos^2\theta - 1.4 \sin^2\theta + 1.9$ Hz, where $\theta = \phi + 60$, and $\phi + 60$.\textsuperscript{27}

* Mean and SD of $\phi$ measured for 10 structures obtained by MD simulations.

C$^{2+}$H of Thr$^1$ and Dab$^2$ NH (data not shown), indicating differential mobility of the linear and cyclic components of PMN. The paucity of NOE cross peaks in the NOESY spectra, a likely consequence of the correlation time of the cyclic portion of PMN being in the zero NOE region, necessitated the ROESY experiments. All intra- and interresidue cross peaks were present in the ROESY spectrum along with a NH/NH NOE between Leu$^6$ and Dab$^7$ (Figure 3a and b). Since PMN has a cyclic array of seven amino acid residues, the possibility of the existence of a $\beta$-turn as proposed for polymyxin B decapeptide by earlier studies in the literature,\textsuperscript{13,14} or a $\gamma$-turn,\textsuperscript{15} were examined. Particular attention was paid to D-Phe$^5$ in view of its propensity to adopt positive $\phi$ angles and therefore to appear in turns.\textsuperscript{39} The characteristic diagnostic NOEs between D-Phe$^5$ C$^{\alpha}$H/Leu$^6$ NH and Leu$^6$/Dab$^7$ NH as well as the solvent inaccessibility of Dab$^7$ NH proton indeed suggest a type II (d,l) $\beta$-turn centered at D-Phe$^5$/Leu$^6$. The relatively low $d\phi/dt$ value for the Dab$^7$ N$^\alpha$H and the NOE between Thr$^9$ C$^{\alpha}$H and Dab$^7$ N$^\alpha$H would be suggestive of a $\gamma$-turn centered around Thr$^9$. The only observable interside-chain NOE was that between the D-Phe$^5$ ring protons and the Leu$^6$ $\delta$-methyl groups. The chemical shifts of the two sets of $\delta$-methyl protons of Leu$^6$ were nonequivalent as mentioned earlier, and the upfield-shifted protons show a more intense NOE with the D-Phe$^5$ ring protons (Figure 3a).

Studies on LPS-Bound PMN: Line Bouddening and TRNOE Experiments

The addition of LPS to aqueous PMN results in a concentration-dependent line broadening of all most all PMN resonances, confirming that exchange between lipid bound and free peptide molecule is fast on the nmr time scale. The side-chain resonances exhibit greater line broadening as compared to the backbone C$^{\alpha}$H and NH resonances (Figure 4). A mixture of LPS and PMN yielding moderately broadened PMN resonances was used for acquiring 2D TRNOE spectra.

In the TRNOE spectrum (Figure 5a and b), the sign of all cross peaks are negative, verifying that the observed cross peaks correspond to the LPS-bound state of the peptide. All backbone NOEs of the cyclic portion of free aqueous PMN (Figure 3a and b) are preserved in the LPS-bound state as well, with an additional NOE between Dab$^8$ NH to Thr$^9$ NH (Figure 5b). However, the intensity as well as the number of backbone-to-side-chain and inter-side-chain NOEs are enhanced remarkably (Figure 5a), suggesting that while the cyclic portion of the peptide undergoes no major backbone conformational change, there is greater motional restriction of the side chains upon binding to LPS. It may be noted that there is a steep dependence of the NOE magnitude on the rotational correlation time in the negative NOE regime.\textsuperscript{40}

New NOE cross peaks that were not observed in the free molecule appear around the turns (Figure 5a and b). In the LPS-bound state, a new backbone NOE between Thr$^9$ NH/Dab$^8$ NH and a side-chain NOE between Thr$^9$-methyl protons/Dab$^7$ N$^\alpha$H are observed. New NOEs are also observed between the side-chain to backbone protons around the $\beta$-turn region. The pair of $\delta$-methyl protons of Leu$^6$ gives NOEs with its NH as well as with Dab$^7$ NH, the two sets of NOEs being of differing intensities. The specific enhancement of these
FIGURE 4  LPS-induced line broadening of PMN resonances. 1D spectrum of 3.5 mM PMN in 90% H₂O/10% D₂O at 298 K, pH 3.0 (Bottom). Escherichia coli LPS added to PMN to a final concentration of 0.3 mg/0.5 mL.
FIGURE 5  (a) 2D TRNOE spectrum of LPS-bound PMN in 90% H$_2$O/10% D$_2$O at 298 K, pH 3.0, 200 ms mixing time. PMN and LPS concentrations are 3.5 mM and 0.6 mg/mL. Sequential assignments of cross peaks between the NH protons to C\(^a\) and side-chain protons are shown. X denotes o,\(\gamma\)-diaminobutyric acid (Dab). (b) 2D TRNOE spectrum of LPS-bound PMN in 90% H$_2$O/10% D$_2$O at 298 K. Assignments of NH/NH cross peaks are shown. Also marked are NOEs between D-Phe\(^{\delta}\) ring protons to its NH and to Leu\(^{\delta}\) NH.
NOEs and the appearance of new NOEs would be consistent with a localized conformational stabilization of the two turns upon complexation with LPS. It may also be noted that the NOEs from the δ-methyl protons to D-Phe\(^5\) 3,4,5 ring protons are stronger than those to D-Phe\(^5\) 2,6 ring protons, indicating a specific orientation of the Leu\(^6\) side chain to the phenyl ring of D-Phe\(^5\).

Enhanced τ\(_r\) value for the peptide side chains will result in more intense NOEs in the complexed state. The Thr\(^1\) C\(_\text{δH}/\text{Dab}\(^2\) NH and Thr\(^1\) methyl/ C\(_\text{αH}\) cross peaks that are present in the free molecule are absent in the LPS-bound form. This observation is consistent with a conformational change involving the Thr\(^1\) side chain upon interaction with LPS.

**Molecular Dynamics**

Molecular dynamics simulations were performed with NOE-derived constraints and H-bond criteria to obtain an ensemble of structures. Examination of the 10 model structures of PMN in water generated by MD superimpose closely (Figure 6), with an rms deviation of 0.30 Å for all the C\(_\text{α}\) atoms suggesting a relative rigidity of the backbone of the cyclic portion. The type II/β-turn around D-Phe\(^3\) and Leu\(^6\) were preserved in all conformers and the γ-turn centered around Thr\(^3\) was also present in all structures; the φ dihedral angle of Thr\(^3\) was invariably around −76°, corresponding to an inverse γ-turn.

The linear moiety was mobile and underwent marked excursions during MD simulation, Dab\(^2\) exhibiting maximal mobility (C\(_\text{α}\) rms: 0.4 Å). The side-chain dihedral (χ) angles are quite variable, as expected. The "stereochemical goodness" of the models were evaluated by plotting the φ,ψ dihedral angles on a Ramachandran map.\(^{41}\) The dihedral angles of all the eight residues fall in the conformationally allowed region in the Ramachandran plot (Figure 6). D-Phe\(^5\) also lies in the stERICALLY allowed region as the φ,ψ map would be inverted upon changing residue chirality. The dihedral angles about the C\(_\text{α}-\text{C\(_\text{α}\)}\) (χ\(_1\)) and C\(_\text{α}-\text{C\(_\text{α}\)}\) (χ\(_2\)) of Dab\(^4\), are approximately 180°. Other Dab residues fall into the extended region of the Ramachandran map whereas Dab\(^8\) adopts a right-handed helical conformation. The two hydrogen
bonds modeled in the crude starting structure (Dab¹⁺: NH ← Dab²⁻: O; Dab³ N⁻H ← Dab⁴⁺: O) were preserved and are of nearly ideal parameters: the N ← O distances are 3.01 and 3.02 Å and the H-N-O angles are 20.9° and 22.5°, respectively.

Figure 7 represents a model of the lipid A-polymyxin B complex that maximizes favorable interactions between the two components. Polymyxin B was generated by the addition of N-methyloctanoyl-Dab residue to the TRNOE-derived PMN model. Docking was achieved interactively with a few cycles of energy minimization to relieve short contacts. The salient features of the complex are the following: The peptide carbonyl groups of the cyclic portion of PMB lie on one face of the peptide, and this surface overlies the disaccharide headgroup of lipid A. The γ-amino groups of Dab³/Dab⁴ and Dab⁵/Dab⁶ are within H-bond distances of the phosphate groups at positions C-1 and C-4' on lipid A, respectively. The orientation of the linear portion of PMB bearing the acyl residue is normal to the plane of the cyclic portion and is coaxial with the acyl chains of lipid A.

DISCUSSION

The conformation of free aqueous PMN has been studied by 2D-nmr methods, and in the LPS-bound state using transferred-NOE techniques in conjunction with molecular modeling methods. While considerable progress has been achieved in understanding the conformational features of cyclic hexa-, octa-, and nonapeptides, relatively little has been reported on cyclic heptapeptides. For this reason, and because the cyclic moiety of PMN is unusual in that it is composed of 23 atoms (due to side-chain-to-main-chain cyclization rather than the 21 in the case of main-chain-to-main-chain linkage), PMN is interesting from the conformational point of view. PMN is also distinguished by the presence of the unusual Dab residue, a feature shared by other B. polymyxa derived antibiotics such as the circlins and octapeptins.

The aqueous conformation of PMN is characterized by a type II' β-turn with D-Phe³ at the i + 1 and Leu⁶ at the i + 2 position identified by the diagnostic NOE pattern and H bonding, and an inverse γ-turn centered at Thr⁹ as evidenced by solvent shielding of Dab¹ N⁻H and the presence of NOEs from the neighboring residues. The remaining portion of the ring is extended without strong transannular H bonds, which is consistent with results obtained by tritium-hydrogen exchange experiments.

The LPS-bound conformation of PMN as examined by TRNOE experiments indicate no major conformational change of the backbone of the cyclic portion, and both turns are preserved. This is consistent with earlier reports showing that the binding of polymyxin to phospholipids do not result in any change in the optical rotatory dispersion spectrum or the amide bond stretching frequencies in the ir spectrum of the peptide. The segregation of the hydrophobic side chains of D-Phe³ and Leu⁶ and the disposition of the carbonyl groups arrayed on one face of the peptide renders the molecule amphiphilic. A model of the PMB-lipid A complex constructed by docking a model of PMB deduced from the TRNOE-derived PMN structure on lipid A suggests that the polar face of PMB interacts with the disaccharide headgroup of lipid A, the hydrophobic side chains of D-Phe³/Leu⁶ overhang-
ing the surface. The $\gamma$-NH$_2$ pairs of the Dab residues Dab$^7$/Dab$^4$ and Dab$^7$/Dab$^8$ form ionic H bonds with the lipid A phosphates. The linear part of PMB is so oriented as to facilitate hydrophobic interactions with the acyl chains of lipid A.

Our investigations were motivated by the assumption that a detailed characterization of the conformation of polymyxin would enhance our understanding of how the peptide functions as an antibiotic, and more importantly, as an LPS antagonist. It would therefore be appropriate to attempt to rationalize polymyxin activity in terms of its structure. The antimicrobial action of polymyxin B is a manifestation of the binding of the peptide to, and subsequent disruption of, the outer membrane of gram-negative bacteria.$^{19}$ The outer monolayer of the outer membrane is comprised almost exclusively of LPS$^{2,24}$ and the initial event of the peptide action, therefore, is its binding to LPS. The presence of anionic constituents (lipid A phosphates and anionic sugar residues), and the highly positively charged nature of polymyxin due to the preponderance of the basic Dab residue, suggest that the binding is electrostatically driven. Indeed, the binding of polymyxin to LPS is inhibited by divalent cations or under conditions of high ionic strength.$^{49}$ LPS obtained from polymyxin-resistant bacterial strains whose lipid A phosphates are esterified show decreased polymyxin binding.$^{53}$ Furthermore, acetylation of the Dab $\gamma$-amino groups of PMB result in loss of antimicrobial activity.$^{32}$ However, while the presence of the basic amino functions are necessary, they are not sufficient for complete expression of activity.

PMN, lacking a single terminal Dab residue covalently linked to a fatty acyl residue, while binding lipid A with a dissociation constant indistinguishable from that of PMB (S. A. David, unpublished results), shows negligible antimicrobial efficacy.$^{17}$ and considerably less potent LPS-antagonistic behavior.$^{33}$ These suggest that microbicidal and anti-LPS activities may have the same structural correlates, the cyclic portion conferring the ability to bind lipid A, and the linear fragment aiding the disruption of LPS assemblies.

The strict structural requirements of the cyclic portion for biological activity were recognized during chemical synthesis of PMB.$^{54}$ These authors reported that the expansion of the cyclic peptide ring by one amino acid reduced antibiotic activity. No major change in the backbone conformations of PMN on LPS binding would therefore suggest that the cyclic ring backbone serves only as a scaffold to appropriately position the Dab side chains, and that the conformation of the ring per se may be relatively unimportant. This conjecture is supported by our earlier findings that gramicidin S and tyrocidin A, basic cyclic decapeptides with rigid $\beta$-pleated structures obtained from B. brevis, also bind lipid A with binding constants comparable to that of PMB$^{35}$; furthermore, subsequent studies have shown that small, linear dibasic nonpeptide molecules (aliphatic diamines and aromatic imidines) of appropriate dimensions also bind lipid A.$^{36}$ The salt bridges observed in modeling the PMB–lipid A complex are likely to be responsible for the high affinity of the peptide for lipid A. The formation of such ionic hydrogen bonds appears to be an obligatory requirement for the interaction of PMB with anionic ligands, since the peptide binds to anionic phospholipids possessing a free phosphate group, but not to analogues with electrostatically similar N-methylated phosphate groups$^{57}$ and poorly to lipid A bearing esterified phosphates.$^{51}$ Interestingly, a variety of cationic organic compounds possessing free protonatable basic groups$^{50}$ bind to lipid A, but not molecules with quarterized nitrogen atoms.

The penetration of the fatty acid tail into the hydrophobic regions of lipid A observed in the model is consistent with increased surface pressure of lipid A monolayers in film balance experiments.$^{38}$ It is likely that the insertion and subsequent appositioning of the hydrophobic component of the peptide destabilizes LPS assemblies on bacterial membranes. It also appears possible that the fatty acyl moiety may mask regions on the hydrophobic domain of lipid A, thereby impeding the recognition of lipid A by its receptors. It has recently been reported that the hydrophobic domain of lipid A is crucial for cellular activation.$^{48}$

These results provide a first step toward an understanding of the structure–activity relationships of polymyxin B, and also permits a greater appreciation of the desirable structural requirements of potential endotoxin antagonists and may be of value in designing analogs of polymyxin with greater LPS-antagonistic properties and lower toxicity.

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