Surface Plasmon Resonance Studies Resolve the Enigmatic Endotoxin Neutralizing Activity of Polymyxin B*  

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Polymyxin B (PMB), a cyclic cationic peptide antibiotic, despite its severe side effects continues to occupy a premiere position for treating endotoxosis. Its mode of neutralization of endotoxin has remained elusive for the last three decades. Several synthetic peptide mimics of PMB, capable of binding endotoxin, have been made. However, the binding ability alone appears to be a deceptive indicator of endotoxin neutralizing activity as molecules with similar binding propensities could either sequester or opsonize the toxin. Hence identification of additional physical parameters which describe adequately the outcome of PMB-endotoxin interaction become imperative. Surface plasmon resonance (SPR) studies reported here show that several mimics of PMB despite exhibiting lipopolysaccharide binding affinities comparable with it but, unlike it, do not sequester the endotoxin. These studies thus provide a striking illustration of the difference in the behavior of PMB, vis a vis its mimics toward the endotoxin lamellae, and define further, in chemical terms, mechanism of the action of PMB and allow us to posit that the design of molecules as effective antidotes for sepsis should incorporate the ability to sequester endotoxin specifically.

Release of miniscule (nanomolar) quantities of lipopolysaccharide (LPS),† the major structural component of the Gram-negative bacterial outer membrane, in systemic bacterial infections frequently leads to a relatively common but often fatal constellation of symptoms termed as the endotoxic shock (1, 2). The treatment for endotoxic shock which is characterized by deranged hemodynamics, coagulation abnormalities, and multiple system organ failure, continues to remain nonspecific and supportive because of the absence of specific interventional strategies (3). However, the mechanisms by which endotoxin (LPS) acts on the target cells are increasingly being understood, which in turn has led to the development of several experimental approaches for treating endotoxosis. These include sequestration of LPS by peptides or anti-LPS antibodies (4–7), use of its antagonistic homologs that prevent its binding to the target cells (8), or the molecules that abrogate signaling to the pathways leading to the production of inflammatory cytokines such as tumor necrosis factor, interleukin-1, etc. (9). Despite these many interventional modalities and the severe side effects associated with the use of polymyxin B (PMB), a cyclic cationic peptide antibiotic, for the treatment of endotoxosis, it continues to occupy the premiere position in our armamentarium for combating endotoxosis (3, 4). Only recently have its mode of interaction with LPS and the structural features involved therein been elucidated (6, 10–12). These and earlier studies have led to the proposal that the asymmetric distribution of the basic and nonpolar groups in polymyxin B impart to it an amphiphilicity that is both necessary and sufficient for its LPS neutralizing activity. That this is indeed the case was proven by a subsequent study with a synthetic linear peptide which exhibits no structural similarity to PMB and in which lysines and nonpolar residues were segregated at either ends of the molecule, endowing it with amphiphilicity sufficient for relatively strong binding to LPS (13). Despite these seeming similarities in the mode of interaction between PMB and other peptides with LPS, binding ability alone appears to be a deceptive indicator of endotoxin neutralizing activity as molecules with nearly the same binding propensities could either opsonize or sequester the toxin (14, 15). It, therefore, becomes imperative to identify alternate or additional physical parameters of interaction which may adequately describe the outcome of the recognition of LPS on its biologic activity, as they may aid in the design and screening of molecules with anti-endotoxic activity. These studies are motivated by such a consideration.

Surface Plasmon Resonance (SPR) studies reported here show that several mimics of PMB despite exhibiting LPS binding affinities comparable with it but, unlike it, do not sequester the endotoxin. We, therefore, consider the removal of endotoxin as a good descriptor of the efficacy of the anti-endotoxic activity of a given compound and suggest inclusion of this criteria in the design and screening of anti-septics.

EXPERIMENTAL PROCEDURES

Materials—PMB sulfate, polymyxin B nonapeptide (PMBN), and fluorescein-labeled lipopolysaccharide (Escherichia coli, 55:B5) were obtained from Sigma. Diphosphoryl lipid A was a product of List Biologicals. Fluorescein-labeled LPS (FITC-LPS) repurified on a Sephadex G-200 column had 8 µg of fluorescein/mg of LPS and exhibited an emission maximum at 520 nm (excitation 495 nm). Wang resin, Fmoc, Fmoc derivatives of amino acids were obtained from Nova-Biochem. All other chemicals used were of the highest purity available.

Peptide Synthesis—The peptides were synthesized on a NovaSyn solid-phase peptide synthesizer using standard Fmoc and Opfp chemistry. After synthesis was completed, the terminal amino group was deprotected from part of the resin. It was then dansylated by flowing dansyl chloride in 10 mM triethylamine at pH 8.1 for 15 min. The peptides were cleaved from the p-hydroxymethylphenoxy polystyrene resin using trifluoroacetic acid containing 5% water (Mili Q, Systems) and 1.5% 1,2-ethanedithiol. The sulphydryl bridge in the decapeptide...
was introduced according to the method of Shih-Yi et al. (16). Peptides were purified on a reverse phase C-18 high performance liquid chromatography column (acetonitrile:water with 0.1% TFA gradient), and their purity was checked with a KRATOS matrix-assisted laser desorption ionization system. The masses of the cyclic decapetide, BPI derived 28-mers peptide, and the 23-mer peptide were 1229.9, 3407.9, and 2523.7, respectively, and their N-α-dansylated counterparts were 1258.3, 3642.8, and 2752.5, respectively. The dendrimeric peptide had a mass of 1094.3.

Quantification of Endotoxin and Peptides—Lipid A samples were quantified by the Limulus amoebocyte lysate assay in pyrogen-free water, using LPS from EndosafeTM as the standard according to the method of Yin et al. (17). Concentrations of the peptides were determined by amino acid analysis using Waters Pico-TagTM systems, whereas that of PMB and PMBN were determined by molar absorbance and weight, respectively (11).

Stopped-flow Spectrofluorimetry—Fast reaction kinetic experiments in the fluoresence mode were performed on an Applied Photophysics SX.18MV stopped-flow apparatus equipped with ARCON 5000 RISC software. The dead time of the instrument was measured to be 1.2 ms. For N-α-dansyl-peptide-LPS interactions, the samples were excited at 340 nm, and emission was monitored beyond 420 nm, by using a cutoff filter, at right angles to the excitation beam. All measurements were made in PBS (50 mM sodium phosphate, pH 7.2, containing 150 mM NaCl) at 20 °C (± 0.1 °C). All traces are cumulative average of ten successive kinetic profiles. Stopped-flow traces were analyzed for mono- and bi-exponential reactions by curve-fitting using the Marquardt algorithm based on the routine curves.

Preparation of Liposomes, Monolayer Deposition on Alkanethiol Chip, and Tethering of Biotinylated Dimyristoylphosphatidylethanolamine Liposomes on Streptavidin Chip—DMPC (1 mM) in 2:1 chloroform/methanol was dried in glass vials under nitrogen and further dried in vacuum for 2 h. PBS, 3 ml, containing varying proportions of lipid A (10–250 μg) was then added to each vial. Vials were then vortexed vigorously for 10 min at 25 °C and sonicated for 30 s in a probe sonicator. Suspension containing liposomes were extruded about 20 times through a 50 nm polycarbonate filters. Liposomes were separated from the unincorporated material by passing through a 25-ml Sepharose CL-6B column. Liposomes with biotinylated phosphatidylethanolamine and lipid A were also prepared as above with final composition of such liposomes being 88% DMPC, 10% lipid A, and 2% biotinylated phosphatidylethanolamine. Alkanethiol (H18TM) chip was cleaned with octylglucoside, and soon thereafter the liposomes as prepared above were passed over it at a flow rate of 2 μl/min for 30 min and washed with PBS. Flow of liposomes followed by PBS was repeated several times until no increase in RU’s was noted. The chip was then washed with a 5-s pulse (10 μl/min) of 20 mM NaOH to remove the multilamellar structures to obtain stable base line. The monolayers thus obtained were dried at a flow rate of 10 μl/min (20 mM PBS) for 10 min. The surfaces are henceforth referred to as “linox monolayers.” Lipid A (1 mg) dissolved in PBS (1 ml), heated at 60 °C for 10 min, and sonicated in a probe sonicator for 5 min was also flown over HPA chip at 2 μl/min for 500 s and washed with PBS for 60 s successively till a maximum of RUs was attained, followed by washes with 20 mM NaOH till stable baseline was achieved as described above. The above surface is henceforth referred to as “neat lipid A monolayers.” Biotinylated-phosphatidylethanolamine/DMPC/lipid A vesicles were passed over the streptavidin sensor chip (Amersham Pharmacia Biotech) at a flow rate of 2 μl/min for 1 h followed by a wash with PBS at a flow rate of 5 μl/min for 20 min. These surfaces are designated as “tethered liposomes with lipid A.”

SPR Analysis—Kinetics of the interaction of the peptides were determined by the SPR using BiaCoreTM biosensor system at 25 °C at a flow rate of 10 μl/min for the determination of on-rates. Off-rates were measured in the dilution mode by flowing PBS at 10 μl/min. The rate constants were determined by the nonlinear least squares fitting of the primary sensogram data using the BiaEvaluation, Version 3.0, software.

Isothermal Titration Calorimetry—A typical titration involved 15–20 injections at 3-min intervals, 4-μl aliquots of peptide solution into the sample cell (volume 1.344 ml) containing lipid A (50 μM), conducted on an OMEGA microcalorimeter of MicroCal, Inc. (11). The titration cell was stirred continuously at 400 rpm. The heat values of the dilution of the peptides in the buffer alone were subtracted from the titration data. The resulting data were then analyzed to determine the binding stoichiometry (n), association constant, and the enthalpy change (ΔH0) as described earlier.

FIG. 1. SPR sensograms and the stopped-flow spectrofluorimetry of cyclic peptide-lipid A interaction. A, monolayer of lipid A (5%) in DMPC matrix on the HPA surface was formed as described in the text (17). B, sensograms indicate the association and dissociation phases of the reactions when cyclic peptide (500, 400, 300, 200, 100 nm, top to bottom) was flown at 10 μl/min over the monolayers containing 5% lipid A. Cyclic peptide did not bind nonspecifically to the DMPC monolayer, but the cyclic peptide/lipid A complex was stabilised at lipid A (1 μM). The fit yield k1 and k−1 as 4.7 × 10^6 M^−1 s^−1 and 0.979 s^−1, respectively. The sensograms fitted to the mass transport limited kinetic analysis also yielded similar results.

RESULTS AND DISCUSSION

Recently we have demonstrated that the interaction of dansyl-PMB with LPS/lipid A consists of a pair of kinetically distinguishable association and dissociation reactions (12). More specifically, the second phase of the association reaction (k2) was ascribed to the insertion of the hydrophobic aspects of PMB into the nonpolar interior of the LPS lamellar phase, which as expected is absent for PMBN which lacks the terminal 6-heptanoyl/octanoyl-χ-diaminobutyryl unit (12). Inevitably, several peptides synthesized for countering endotoxic shock display, unlike PMB, monophasic kinetic behavior as exemplified by the stopped-flow kinetic analyses of the binding of the N-α-cyclic decapetide (Fig. 1, Table I, and Scheme I).

Study of macromolecule-ligand interaction by SPR method depends solely on the mass changes during the reaction (18, 19). Additionally, in SPR the ability to form model membrane assemblies, monolayers, or bilayers incorporating the biologic...
SPR data are from an average of four experiments. The rate constants evaluated by the stopped-flow spectrofluorimetry are given in parentheses (12) (averages of 10 with errors < 10%). $K_{\text{app}}$ for PMB (11) and that from kinetic experiments (12) are $1.8 \times 10^{5}$ M$^{-1}$ and 2.3 $\times 10^{3}$ M$^{-1}$, respectively. $k_{1}$ and $k_{-1}$ of I and II and the lipid A reported here are close to those determined by passing the LPS over the peptides (1 and 2) immobilized covalently on CM5 chip (21, ND), not done.

| Peptide | $k_{1} \times 10^{5}$ | $k_{-1}$ | $K_{a} \times 10^{5}$ | $K_{\text{app}} \times 10^{5}$ |
|---------|----------------------|----------|---------------------|---------------------|
| II      | 2.4 (2.9)            | 0.084 (0.097) | 2.85 (2.40) | 3.2 |
| III     | 4.7 (5.1)            | 0.097 (0.131) | 4.84 (3.92) | 5.1 |
| IV      | 3.6 (ND)             | 0.085 (ND) | 4.23 (ND)    | 5.9 |
| V       | 6.8 (7.1)            | 0.091 (0.150) | 7.47 (6.40) | 7.9 |
| VI      | 9.5 (9.8)            | 0.076 (0.091) | 12.5 (11.10) | 11.6 |

| Peptide | $K_{b} \times 10^{5}$ |
|---------|----------------------|
| X-DAB-Thr-DAB-DAB-DAB-DAB-PA | 5.1 (6.1) |
| OC-Thr-DAB-DAB-Leu | 0.131 (0.133) |
| OC-Thr-DAB-DAB-Leu | 0.131 (0.133) |

**Scheme 1.**

**Table 1. Kinetic and thermodynamic parameters for the interaction of peptides with lipid A determined by SPR, stopped-flow spectrofluorimetry, and FITC at 20 °C.**

Peptide receptors which mimic the natural environment offer additional opportunities to study, in molecular terms, the surface-associated phenomena. A representative sensogram for the interaction of cyclic peptide, with the monolayers of 5% lipid A incorporated in L-$\alpha$-phosphatidylcholine, dimyristoyl (DMPC) matrix show rapid increase in RUs (Fig. 1). These changes in RUs correspond to $k_{1}$ and $k_{-1}$ of 4.7 $\times 10^{5}$ M$^{-1}$ s$^{-1}$ and 0.097 s$^{-1}$, respectively. Residuals confirm further the monoeponential nature of the reaction (data not shown). The values of $k_{1}$ and $k_{-1}$ for the dansyl-cyclic peptide-lipid A interaction determined by the stopped-flow method and shown as a representative example are 5.1 $\times 10^{4}$ s$^{-1}$ and 0.131 s$^{-1}$, respectively. Good agreement between the rate constants determined by SPR with those obtained by the stopped-flow experiments lends further confidence to our SPR data (Fig. 2, Table 1). Unexpectedly, however, the SPR analyses of the binding of PMB with lipid A/DMPC monolayers showed a time- and concentration-dependent diminution in the RUs (Fig. 2, A). The time-dependent drop in RUs indicates that PMB is able to "take off" some mass from such monolayers. Experiments with monolayers made with DMPC alone did not show any changes in RUs ruling out the interaction of PMB with the phospholipid and its sequestration from the surface. Conversely, a time- and PMB concentration-dependent drop in RUs from neat lipid A monolayers was also observed (Fig. 2B). These experiments thus leave us with the inescapable conclusion that, as opposed to other peptides examined, PMB is able to from a specific complex with endotoxin and sequester it. Our failure to observe the initial rise for PMB could be related to alteration of the chemical environment of the surface which causes the signal to drop because of a conformational change in the immobilized surface. Nevertheless, appearance of FITC-LPS in the flow-through of SPR experiments done with PMB and not in experiments with other peptides is consistent with the removal of the endotoxin from the immobilized surface (data not shown).

We have also used liposomal preparations containing lipid A (10%) tethered to streptavidin immobilized on the biosensor chip through biotinylated phosphatidylethanolamine (2%) in 88% DMPC and noted that, among the peptides studied, PMB alone manifests a drop in RUs from such bilayer vesicles (Fig. 2A). This decrease in RUs as a function of time reflects a specific removal of the endotoxin from the vesicles as the liposomes without endotoxin fail to suffer any diminution in RUs. The apparent rate constant for the removal of the endotoxin from the neat lipid A, DMPC/lipid A monolayers and from the tethered liposomes with lipid A are nearly the same (0.042–0.046 s$^{-1}$); B, removal of lipid A from the tethered liposomal bilayer. The peptides were injected over the tethered liposomes (containing 10% lipid A) which yielded $k_{1}$ and $k_{-1}$ values similar to the monolayer experiments. 28-residue peptide binds to lipid A and displays typical association and dissociation reactions (b), whereas PMB takes off the lipid A from liposomes with similar rates as that from the monolayers of DMPC/lipid A (c). Experiments (1–4) were conducted as a function of 25, 50, 75, and 100 nm of PMB, respectively.

![FIG. 2. Take off of the lipid A from lipid A/DMPC monolayers from the chip by PMB. A, PMB flown (10 μm/min) over these monolayers "lifts off" lipid A as a function of concentration (curves 1–4 are with 2, 5, 10, and 25 mol % of lipid A, respectively, in DMPC). PMB is also able to remove lipid A in a concentration-dependent manner (curves 5, PMB = 125 nm; and curve 6, PMB = 300 nm) from a neat monolayer formed entirely of lipid A. The apparent rate constants for the removal of lipid A from the DMPC-containing, as well as the neat monolayers, is 0.042–0.049 s$^{-1}$, B, removal of lipid A from the tethered liposomal bilayer. The peptides were injected over the tethered liposomes (containing 10% lipid A) which yielded $k_{1}$ and $k_{-1}$ values similar to the monolayer experiments. 28-residue peptide binds to lipid A and displays typical association and dissociation reactions (b), whereas PMB takes off the lipid A from liposomes with similar rates as that from the monolayers of DMPC/lipid A (c). Experiments (1–4) were conducted as a function of 25, 50, 75, and 100 nm of PMB, respectively.](image-url)
so: (i) by altering the organization of the endotoxin in the lamellar phase, (ii) by coating the LPS lamellar phase, and/or (iii) by solubilizing and removing it from the LPS assembly. Our SPR studies show clearly that PMB is very effective in solubilizing endotoxin from its assembly. Conversely, a number of peptides that interact with the endotoxin fail to do so.

At this stage it is instructive to consider these SPR data in conjunction with the analyses of PMB-lipid A/LPS interactions by stopped-flow spectrofluorimetry (12). As alluded to earlier, the stopped-flow experiments explicitly show that the bi-molecular association between PMB and LPS is followed by the insertion of the hydrophobic parts of the antibiotic in the apolar milieu of the endotoxin lamellar phase which, as expected, is described well by a unimolecular reaction. It, therefore, appears likely that the endotoxin “take off” in SPR experiments is synonymous mostly, if not entirely, with the unimolecular phase of the spectroscopically monitored reaction, whereas the bimolecular phase of the spectroscopically determined reaction ($k_1$) is kinetically indistinguishable from that determined by SPR by monitoring the interaction between lipid A and the covalently immobilized PMB (21). This also appears quite likely from the fact that, under a variety conditions (such as the variation of PMB concentration), the rate of the removal of endotoxin from lipid A/DMPC and or neat lipid A monolayers or the tethered liposomal bilayers remains invariant.

In summary, these studies provide a striking illustration of the difference in the behavior of PMB vis a vis its mimics toward the endotoxin lamellae and define further, in chemical terms, mechanism of the action of PMB which allows us to posit that the design of molecules as effective antidotes for sepsis should incorporate the ability to remove endotoxin specifically.

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