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Characterization of the Structural Elements in Lipid A Required for Binding of a Recombinant Fragment of Bactericidal/Permeability-Increasing Protein $rBPI_{23}$

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Both human bactericidal/permeability-increasing protein (BPI) and a recombinant amino-terminal fragment of BPI ($rBPI_{23}$) have been shown to bind with high affinity to the lipid A region of lipopolysaccharide (LPS) (H. Gazzano-Santoro, J. B. Parent, L. Grinna, A. Horwitz, T. Parsons, G. Theofan, P. Elsbach, J. Weiss, and P. J. Conlon, Infect. Immun. 60:4754–4761, 1992). In the present study, lipid A preparations derived from bacterial LPS as well as synthetic lipid A's and various lipid A analogs were used to determine the structural elements required for $rBPI_{23}$ binding. $rBPI_{23}$ bound in vitro to a variety of synthetic and natural lipid A preparations (both mono- and diphosphoryl forms), including lipid A's prepared from *Escherichia coli* and *Salmonella*, *Neisseria*, and *Rhizobium* species. Binding does not require that the origin of negative charge be phosphate, since $rBPI_{23}$ bound with high affinity to lipid A's isolated from *Rhizobium* species that contain carboxylate (*Rhizobium trifolii*) or sulfate (*Rhizobium meliloti*) anionic groups and lack phosphate. Lipid A acyl chains are important, since $rBPI_{23}$ did not bind to four synthetic variants of the β(1-6)-linked D-glucosamine disaccharide lipid A head group, all devoid of acyl chains. $rBPI_{23}$ also bound weakly to lipid X, a monosaccharide lipid precursor of LPS corresponding to the reducing half of lipid A. Lipid IV_A, a precursor identical to *E. coli* lipid A except that it lacks the 2′ and 3′ acyl chains, was the simplest structure identified in this study that $rBPI_{23}$ bound with high affinity. These results demonstrate that $rBPI_{23}$ has a binding specificity for the lipid A region of LPS and binding involves both electrostatic and hydrophobic components.

Lipopolysaccharides (LPS, endotoxins) are major components of the outer membrane of gram-negative bacteria and have been implicated in the clinical syndrome of gram-negative bacterial septic shock (26, 29, 30). Chemically, LPS are composed of a poly- or oligosaccharide and a lipid component, termed lipid A. Lipid A is the most conserved part of the LPS structure and represents the endotoxic principle of LPS (31). The backbone of lipid A in a number of bacterial families, including *Enterobacteriaceae*, is composed of a β(1-6)-linked D-glucosamine disaccharide with glycosidically bound phosphate attached at position 1 and an ester-bound phosphate attached at position 4′ (Fig. 1). The hydrophilic backbone is acylated by four to seven ester- or amide-linked fatty acid residues, with carbon chain lengths of usually 12 to 16 carbon atoms.

Bactericidal/permeability-increasing protein (BPI), a naturally occurring bactericidal protein found in neutrophil granules, is highly cytotoxic toward gram-negative organisms (6, 7, 33, 39, 40). This target cell specificity is attributable to the strong interaction of BPI with LPS in the bacterial envelope (21, 38, 40, 41). We have previously reported that BPI binds with high affinity (K_d of 2 to 5 nM) to lipid A and a wide spectrum of LPS isolated from clinically relevant bacterial strains (10). Importantly, BPI also inhibits biological responses to LPS (22, 23, 25, 27).

BPI's potent bioactivities have been shown to reside in the amino-terminal portion of the holo-protein (20, 27, 28). A recombinant amino-terminal fragment derived from BPI, designated $rBPI_{23}$, displays bactericidal and LPS-neutralizing properties similar to those of the 55-kDa holo-protein both in vitro (3, 9, 15, 24, 25, 40) and in vivo (1, 16, 17, 19). Furthermore, the binding properties of $rBPI_{23}$ and the holo-protein for lipid A and LPS are essentially identical (10), and $rBPI_{23}$ binds to an extensive panel of LPS isolated from clinically relevant gram-negative bacteria (2).

Although $rBPI_{23}$ has been shown to recognize the highly conserved lipid A region of bacterial LPS, little is known about the structural features of lipid A that are important for high-affinity binding by $rBPI_{23}$. In this study, we have evaluated the influence of phosphate, the number of fatty acid chains, and the structure of the lipid A backbone on $rBPI_{23}$ reactivity.

MATERIALS AND METHODS

Reagents. *Salmonella minnesota* R595 lipid A (monophosphoryl and diphosphoryl) and *Escherichia coli* J5 (Rc) lipid A (diphosphoryl) were obtained from commercial sources (List Biological Laboratories, Inc., Campbell, Calif., and Ribi ImmunoChem Research, Inc., Hamilton, Mont.). Lipid A from *Rhizobium* strains was isolated and purified as described earlier (12, 14). Lipid A from *Neisseria meningitidis* was purified as previously described (18). Synthetic tetraacyl 1,4′-diphosphoryl lipid A precursor IV_A (LA-14-PP) was purchased from ICN Biomedicals, Inc., Costa Mesa, Calif.). This structure is designated according to the nomenclature of Takada and Kotani (34). Lipid X was obtained from Lipidex, Inc., Middleton, Wis. The other synthetic disaccharide lipid A partial structures, GlcNac-β(1-6)-GlcNac, GlcNac-β(1-6)-GlcNac-1-phosphate, GlcNac-β(1-6)-GlcNac-4′-phosphate, and GlcNac-β(1-6)-GlcNac-1,4′-phosphate, were prepared by direct synthesis. Details of the synthesis will be published elsewhere. Monosialoganglioside-G_{M1}, phosphatidylglycerol, and phosphatidylserine were purchased from Sigma Chemical Company. Bovine serum albumin (BSA; very

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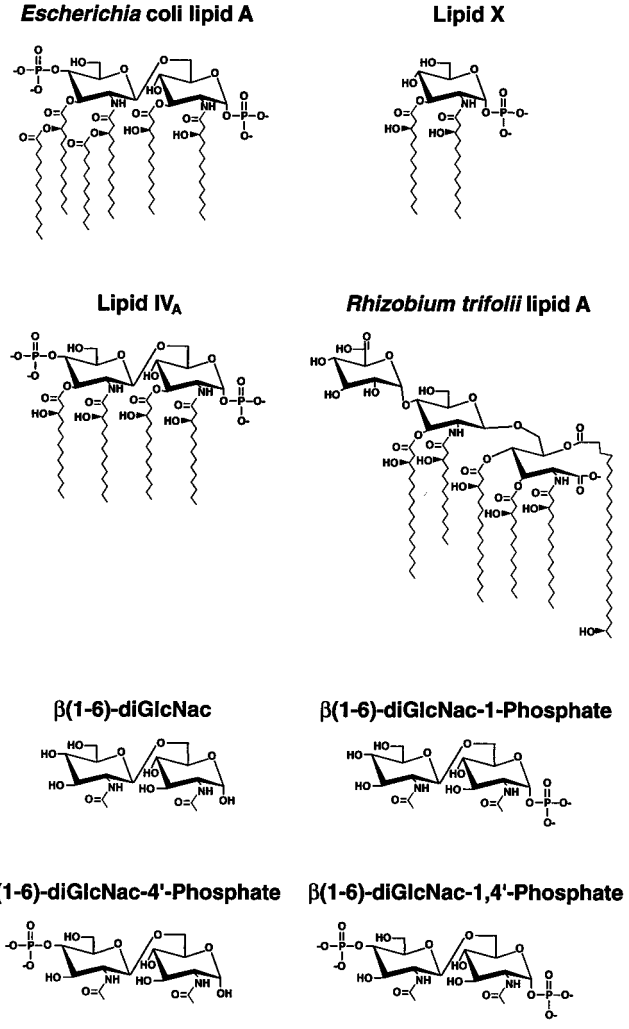

FIG. 1. Chemical structures of *E. coli* J5 lipid A, lipid IV_A, lipid X, GlcNac-β(1-6)-GlcNac, GlcNac-β(1-6)-GlcNac-1-phosphate, GlcNac-β(1-6)-GlcNac-4′-phosphate, and GlcNac-β(1-6)-GlcNac-1,4′-phosphate and *R. trifolii* lipid A proposed by Bhat et al. (4). There is considerable heterogeneity in the fatty acid distribution of the *R. trifolii* lipid A structure.

low endotoxin [less than 0.2 endotoxin units/mg of BSA]; fatty acid free) was purchased from Miles, Kankakee, Ill.

$rBPI_{23}$ purification. $rBPI_{23}$ was cloned, expressed from a construct encoding the first 199 amino acids of BPI, and purified to homogeneity by using standard techniques (10).

Preparation of ^{125}I-labeled $rBPI_{23}$. Protein radiolabeling was carried out by using Iodo-Beads (Pierce Chemical Co., Rockford, Ill.) and carrier-free $Na^{125}I$ as previously described (10). The specific activity of ^{125}I-labeled $rBPI_{23}$ varied between 5 and 16 μCi/μg in different preparations.

Inhibition of radiolabeled $rBPI_{23}$ binding to immobilized *E. coli* J5 (Rc) lipid A. *E. coli* J5 (Rc) lipid A was sonicated, diluted in absolute methanol to a concentration of 0.5 μg/ml, dispensed in 50-μl aliquots into wells (Immulon 2 Removawell Strips; Dynatech, Chantilly, Va.), and evaporated overnight at 37°C. Wells were then blocked with 0.1% very-low-endotoxin BSA prepared in Dulbecco's phosphate-buffered saline (PBS) (Ca^{2+}- and Mg^{2+}-free) for 3 h at 37°C and washed twice in Dulbecco's PBS (Ca^{2+} and Mg^{2+} free) containing 0.05% Tween 20. The blocking solution was discarded and 50-μl aliquots of ^{125}I-labeled $rBPI_{23}$ (200,000 cpm/50 μl) corresponding to a concentration of 5 to 10 nM were then incubated overnight at 4°C in the presence of increasing amounts of the compound under study. After three washes, the radioactivity remaining in the wells was counted, and the results are reported as the means of six replicate samples. The binding of ^{125}I-labeled $rBPI_{23}$ to wells treated with 0.1% BSA only was taken to represent nonspecific binding; specific binding of ^{125}I-labeled $rBPI_{23}$ was defined as the difference between total and nonspecific binding. Nonspecific binding represented between 5 and 10% of total binding.

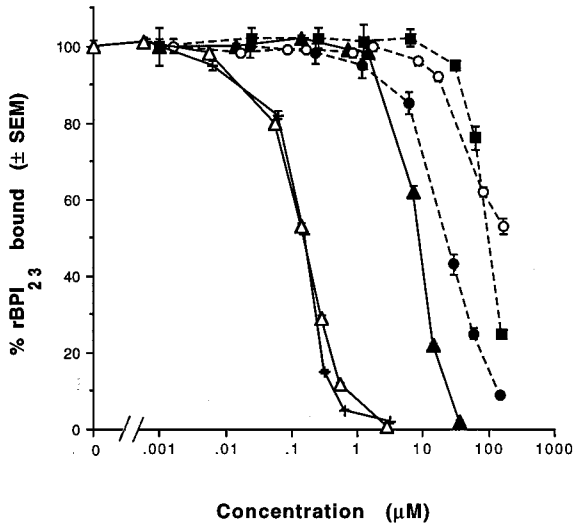

FIG. 2. Inhibition of radiolabeled $rBPI_{23}$ binding to immobilized *E. coli* lipid A by lipid A analogs and selected lipids. Immobilized *E. coli* J5 lipid A was incubated overnight at 4°C in the presence of ^{125}I-labeled $rBPI_{23}$ (10 nM) and increasing concentrations of *E. coli* J5 lipid A (diphosphoryl) (△), LA-14-PP (precursor IV_A) (+), lipid X (▲), phosphatidylserine (●), phosphatidylglycerol (■), or monosialoganglioside-G_{M1} (○) as described in Materials and Methods. Each point represents the mean of six replicates ± standard error of the mean.

All experiments were conducted at least three times, and representative results are given.

RESULTS

Inhibition of $rBPI_{23}$ binding to immobilized lipid A. Two precursors in the biosynthetic pathway of lipid A are lipid X and lipid IV_A (30). Lipid X is a derivative of D-glucosamine 1-phosphate acylated with two fatty acid chains and corresponds to the reducing half of lipid A (Fig. 1). Lipid IV_A is identical in structure to *E. coli* lipid A except that it lacks the two fatty acid chains attached to the ester-linked 3-hydroxy fatty acids (Fig. 1). To evaluate the ability of $rBPI_{23}$ to recognize these precursor forms as well as other selected lipid A variants, we used a competition assay. In this assay, the glycolipid concentration required to inhibit the binding of ^{125}I-labeled $rBPI_{23}$ to immobilized *E. coli* J5 lipid A by 50% (IC_{50}) was determined. Figure 2 shows that synthetic lipid X was a relatively weak inhibitor in the competition assay; the IC_{50} for lipid X was approximately 70-fold higher than the IC_{50} for *E. coli* lipid A (Table 1). In contrast, the IC_{50}s for LA-14-PP, a

TABLE 1. Binding specificity of $rBPI_{23}$

Substance[a]	IC_{50} (μM)
R. meliloti lipid A	0.12
S. minnesota R595 lipid A (DP)	0.15
E. coli J5 (Rc) lipid A (DP)	0.15
LA-14-PP (precursor IV_A)	0.15
N. meningitidis lipid A	0.25
S. minnesota R595 lipid A (MP)	0.6
R. trifolii lipid A	1
Lipid X	10
Phosphatidylserine	28
Phosphatidylglycerol	100
Monosialoganglioside-G_{M1}	>100

[a] DP, diphosphoryl; MP, monophosphoryl.

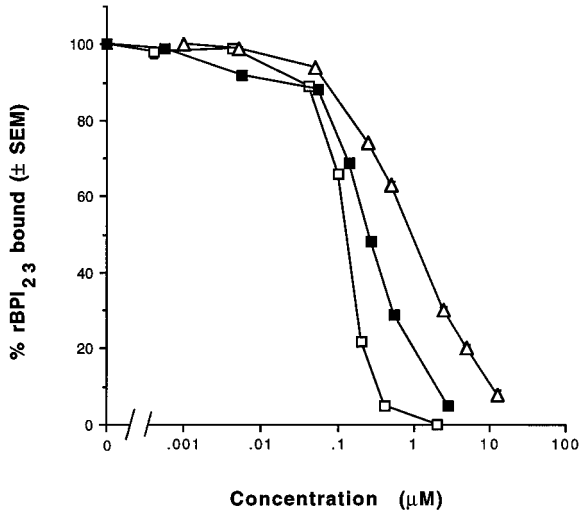

FIG. 3. Inhibition of radiolabeled $rBPI_{23}$ binding to immobilized *E. coli* lipid A by atypical lipid A forms. Immobilized *E. coli* J5 lipid A was incubated overnight at 4°C in the presence of ^{125}I-labeled $rBPI_{23}$ (10 nM) and increasing concentrations of lipid A from *R. meliloti* (□), *R. trifolii* (△), or *N. meningitidis* (■) as described in Materials and Methods. Each point represents the mean of six replicates ± standard error of the mean.

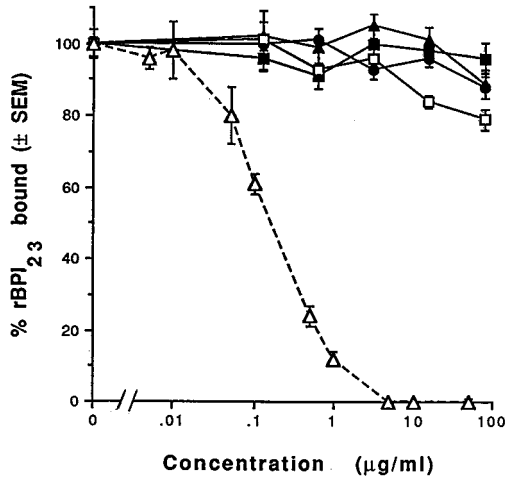

FIG. 4. Effects of four synthetic variants of the β(1-6)-D-glucosamine disaccharide lipid A head group on binding of radiolabeled $rBPI_{23}$ to immobilized *E. coli* lipid A. Immobilized *E. coli* J5 lipid A was incubated overnight at 4°C in the presence of ^{125}I-labeled $rBPI_{23}$ (6.5 nM) and increasing concentrations of *E. coli* J5 (Rc) lipid A (△), GlcNac-β(1-6)-GlcNac (●), GlcNac-β(1-6)-GlcNac-1-phosphate (■), GlcNac-β(1-6)-GlcNac-4′-phosphate (▲), or GlcNac-β(1-6)-GlcNac-1,4′-bisphosphate (□) as described in Materials and Methods. Each point represents the mean of six replicates ± standard error of the mean.

synthetic form of precursor IV_A, and *E. coli* J5 lipid A were the same (Fig. 2). To further evaluate the binding specificity of $rBPI_{23}$, we tested three polar lipids unrelated to lipid A in the competition assay. As shown in Fig. 2, phosphatidylglycerol and monosialoganglioside-G_{M1} were weak inhibitors in this assay, and the IC_{50} for phosphatidylserine was about threefold higher than that of lipid X.

To evaluate the role of lipid A phosphate groups on $rBPI_{23}$ reactivity, we tested a number of lipid A forms with reduced or absent phosphate in the competition assay. As shown in Table 1, the IC_{50} for monophosphoryl lipid A was fourfold higher than that for diphosphoryl lipid A. Contamination of the monophosphoryl lipid A preparation with diphosphoryl lipid A cannot account for the IC_{50} of the monophosphoryl lipid A preparation since proton, carbon-13, and phosphorus-31 nuclear magnetic resonance studies on the monophosphoryl lipid A preparation have demonstrated no diphosphoryl lipid A contamination (37). To further study the role of lipid A phosphate groups, we evaluated the inhibitory activity of *Rhizobium meliloti* lipid A, which has sulfate rather than phosphate attached to the D-glucosamine-containing lipid A backbone (5). *R. meliloti* lipid A was as potent as *E. coli* lipid A in the competition assay (Fig. 3; Table 1). The hydrophilic backbone of *Rhizobium trifolii* ANU 843 lipid A also lacks phosphate but contains carboxylate groups as a source of negative charge (14). A structure for the lipid A of *R. trifolii* and other related rhizobia was recently proposed (4) (Fig. 1). *R. trifolii* lipid A was active in the competition assay, but the IC_{50} for *R. trifolii* lipid A was about six- to sevenfold higher than for *E. coli* lipid A.

N. meningitidis lipid A is unusual since most of the phosphate groups in the lipid A molecules are substituted by *O*-phosphorylethanolamine (18). As shown in Fig. 3, *N. meningitidis* lipid A was able to compete $rBPI_{23}$ binding in a comparable fashion to *E. coli* lipid A.

To evaluate the role of lipid A fatty acids and phosphate on $rBPI_{23}$ reactivity, we synthesized four variants of the β(1-6)-linked D-glucosamine disaccharide lipid A head group, all devoid of fatty acid chains but varying in the presence or absence of C-1 and C-4′ phosphate (Fig. 1). As can be seen in Fig. 4, none of these disaccharides, at concentrations up to 80 μg/ml, inhibited $rBPI_{23}$ binding to lipid A. We also tested a number of common monosaccharide constituents of the LPS core, including 2-keto-3-deoxyoctulosonic acid, L-*glycero*-D-mannoheptose, D-glucose, D-galactose, and *N*-acetyl-D-glucosamine, and they were inactive in the competition assay at concentrations up to 1 mg/ml (data not shown).

DISCUSSION

Our previous studies demonstrated that $rBPI_{23}$ binds with high affinity to the lipid A moiety of LPS (10). In the studies reported here, we have attempted to identify the structural features on lipid A that are required for high-affinity binding by $rBPI_{23}$. The typical lipid A is negatively charged with phosphate attached to the 1 and 4′ hydroxyl groups of the glucosamine disaccharide head group. Therefore, we examined the influence of phosphate on $rBPI_{23}$ reactivity. Using in vitro competition assays, we found that binding of $rBPI_{23}$ to *E. coli* 4′-monophosphoryl lipid A was less than binding to the 1,4′-diphosphoryl form, demonstrating that the presence of glycosidically bound phosphate at the 1 position is important for high affinity binding. Lipid A's isolated from some organisms have charged substituents such as phosphoryl ethanolamine or amino sugars attached to the phosphate groups. For example, both the 1 and 4′ phosphate groups in most (about 85%) of the *N. meningitidis* lipid A molecules are substituted by *O*-phosphorylethanolamine (18). In the competition assay, *N. meningitidis* lipid A was as potent as *E. coli* lipid A, suggesting that $rBPI_{23}$ does not require unsubstituted 1 and 4′ phosphate groups for binding.

We extended our studies to determine if $rBPI_{23}$ has a requirement for phosphate as the anionic group on lipid A. In radiolabeling studies, *R. meliloti* lipid A has been shown to have sulfate rather than phosphate attached to the D-glucosamine-containing lipid A backbone (5). In the competition assay, *R. meliloti* lipid A was as potent as *E. coli* lipid A. The hydrophilic backbone of *R. trifolii* ANU 843 lipid A also lacks

phosphate and is predominantly composed of D-galacturonic acid and D-glucosamine (14). There is also a minor amount of a fatty acylated amino glucuronic acid residues (13). A structure for the lipid A of *R. trifolii* and other related rhizobia in which galacturonic acid, glucosamine aldonic acid, and glucosamine are present in equal amounts was recently proposed (4). Thus, *R. trifolii* is an unusual molecule which lacks phosphate but contains carboxylate groups as a source of negative charge. *R. trifolii* lipid A was found to be active in the competition assay but was six- to sevenfold less potent than *E. coli* lipid A. These results demonstrate that phosphate is not required for $rBPI_{23}$ reactivity and that other negatively charged groups attached to the lipid A backbone may suffice. It is interesting that the *R. trifolii* lipid A is a more potent inhibitor than *R. meliloti* lipid A, and this may be explained by the fact that the carboxylates present in *R. trifolii* LPS are not as ionized as the sulfates present in *R. meliloti*; therefore, the electrostatic component will be less (5).

Lipid IV_A and lipid X correspond to synthetic derivatives of known intermediates of the biosynthesis of lipid A. Lipid IV_A lacks the 2′ and 3′ normal acyl chains but is otherwise identical to *E. coli* lipid A. $rBPI_{23}$ bound to lipid IV_A with high affinity. In contrast, $rBPI_{23}$ bound poorly to lipid X, a monosaccharide lipid A precursor. Synthetic lipid X consists of a glucosamine residue with a phosphate group at the C-1 position and two 3-hydroxytetradecanoic fatty acid chains (34). These results suggest either that the presence of a disaccharide lipid A backbone may be required for high-affinity binding by $rBPI_{23}$ or that more that two fatty acid chains are required for high-affinity binding, or both.

To further investigate the interaction between $rBPI_{23}$ and the lipid A backbone, we tested four versions of the β(1-6)-linked D-glucosamine disaccharide lipid A head group, all devoid of acyl chains, that varied in terms of the presence or absence of C-1 and C-4′ phosphate. In the competition assay, none of these disaccharides inhibited $rBPI_{23}$ binding to lipid A. Since lipid A molecules form supramolecular aggregates in an aqueous environment whereas the synthetic diglucosamine head group variants are water soluble, these results suggest that $rBPI_{23}$ interacts with hydrophobic domains in the fatty acid region of lipid A or $rBPI_{23}$ reactivity requires aggregated lipid A, or both. Since electrostatic interactions are much stronger than hydrophobic interactions, the collective charge on the LPS aggregate array would be a very important component. This would be lost in the absence of fatty acids and may explain why the lipid A head group (minus the fatty acids) is incapable of blocking $rBPI_{23}$ binding to lipid A. Since $rBPI_{23}$ also contains hydrophobic sequences, there should still be some level of interaction between the hydrophobic regions of the two molecules once they are bound. The hydrophobic region of the $rBPI_{23}$ structure might also allow these molecules to form complementary aggregate structures in which the positively charges groups are present as an array. Either way, our results demonstrate that the presence of acyl chains on lipid A influences its interaction with $rBPI_{23}$. Lipid IV_A, a lipid A precursor with four acyl chains was the simplest structure identified in this study that $rBPI_{23}$ bound with high affinity.

Human BPI shares a 45% amino acid sequence identity with another lipid A-binding protein, human LPS-binding protein (LBP) (35). LBP has been reported to bind to precursor IV_A but not to lipid X, similar to our results with $rBPI_{23}$ (36). The two proteins appear to have similar binding specificities for LPS; however, BPI has a significantly higher affinity than LBP for binding to lipid A and gram-negative bacteria (9). The two lipid A-binding proteins also have very different functional activities. In contrast to the endotoxin-neutralizing activity of BPI, LBP is a serum protein that markedly potentiates the sensitivity of the host to LPS by a mechanism which involves binding of the LBP-LPS complex to cell surface CD14 receptors (42). The divergent functional properties of these two lipid A-binding proteins may be explained by the inability of BPI-LPS complexes to bind to CD14 receptors (8).

The crystal structure of an endotoxin-neutralizing protein from the horseshoe crab, *Limulus* anti-LPS factor (LALF), has been reported by Hoess et al. and the authors suggest that BPI and LALF have similar LPS binding motifs (11). LALF is wedge shaped like a slice from a very deep circular cake. It has a packed hydrophobic domain that is perpendicular to the top surface of the slice. The top triangular face has 10 basic residues. This molecule should form aggregate structures that look like LPS aggregates except that their surfaces are positively instead of negatively charged (32). One model for the LPS neutralizing effects of this protein is that the hydrophobic region fuses with the hydrophobic core of the LPS aggregates and the charged top surface interacts with the phosphate groups. The initial interaction is electrostatic. This leads eventually to (membrane) fusion. BPI also has a hydrophobic core and an area containing a high density of basic groups. These similarities between LALF and BPI are pointed out in the report by Hoess et al. (11).

In summary, our results indicate that the interaction between $rBPI_{23}$ and LPS takes place at the level of the lipid A anchor region and that binding involves both electrostatic and hydrophobic components. This mechanism of interaction between $rBPI_{23}$ and LPS is consistent with the fact that $rBPI_{23}$ has hydrophobic stretches which could insert into the hydrophobic core of the outer membrane or LPS aggregate or, more likely, cause $rBPI_{23}$ to form a complementary array. The precise location and arrangement of basic residues (lysine and arginine) on $rBPI_{23}$ allow interaction with negative charges in the LPS lipid A region.

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