Lipopolysaccharide (LPS)-binding proteins BPI and LBP Form Different Types of Complexes with LPS*

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Lipopolysaccharide (LPS)-binding protein (LBP) and bactericidal/permeability-increasing protein (BPI) are closely related LPS-binding proteins whose binding to LPS has markedly different functional consequences. To gain better insight into the possible basis of these functional differences, the physical properties of LBP-LPS and BPI-LPS complexes have been compared in this study by sedimentation, light scattering, and fluorescence analyses. These studies reveal dramatic differences in the physical properties of LPS complexed to LBP versus BPI. They suggest that of the two proteins, only LBP can disperse LPS aggregates. However, BPI can enhance both the sedimentation velocity and apparent size of LPS aggregates while inhibiting LPS-BPI binding even at very low (1:40 to 1:20) BPI:LPS molar ratios.

The lipopolysaccharide (LPS)-binding protein1 (LBP) and the bactericidal/permeability-increasing protein (BPI) are both LPS-interactive mammalian proteins with approximately 45% amino acid sequence identity (1, 2). LPS is considered to be the principal component of Gram-negative bacteria that alerts the host to invading bacteria and triggers defensive responses (3, 4). These responses are usually beneficial and effective but may also become excessive and lead to endotoxic shock (3–5). Both LBP and BPI modulate the bioactivity of LPS (2, 3, 5). LBP is a plasma protein that catalyzes the transfer of LPS from LPS aggregates to other LPS-binding proteins (3, 6–9). Prominent among these is CD14, a surface molecule of myeloid cells that is also present in the circulation as a soluble protein. LBP and CD14 together represent the main pathway by which cells recognize low concentrations of LPS and are stimulated to respond to Gram-negative bacteria (3, 10, 11). In contrast to the LPS-stimulatory properties of LBP, binding of LPS by BPI results in inhibition of the bioactivities of LPS (2). BPI is produced by polymorphonuclear leukocytes and stored in its azurophilic granules (12, 13). It contributes substantially to both the intracellular and extracellular antibacterial activity of polymorphonuclear leukocyte-rich inflammatory exudates toward Gram-negative bacteria (14, 15). The high affinity of BPI for LPS accounts for the target-cell specificity of the antibiotic activity of BPI for Gram-negative bacteria (2, 16). In contrast to BPI, binding of LBP to bacterial envelope LPS does not produce detectable membrane alterations or other antibacterial effects.

We have recently described the use of fluorescein-labeled LPS (FITC-LPS) as well as metabolically labeled ([3H]LPS) and sucrose density gradients to characterize the formation and physical properties of complexes of LPS with LBP and the soluble form of CD14 (7). In this report we describe results obtained by studying the binding of FITC-LPS and [3H]LPS to BPI using the techniques described earlier and in addition compare the effects of LBP and BPI on the light scattering properties of LPS. The results obtained reveal striking differences in the physical properties of LBP-LPS and BPI-LPS complexes. Thus, under conditions in which complexing of LPS with LBP and sCD14 disaggregates LPS, complexing of LPS with BPI appears to modify but not dissolve LPS aggregates. These effects of BPI on LPS aggregates are observed at molar ratios as low as 1 BPI:40 LPS.

EXPERIMENTAL PROCEDURES

Materials—Isolation of native human BPI (17) and LBP (18) and preparation of FITC-LPS (7) and biosynthetically labeled [35S]BPI (19) were carried out as described previously. Recombinant human BPI and LBP were obtained from XOMA Corp. (Berkeley, CA) and used interchangeably with the native proteins. Salmonella minnesota Re595 LPS was obtained from List Biological Laboratories (Campbell, CA). Metabolically labeled [3H]LPS (10,000 cpm/ng) prepared from Escherichia coli LCD25 was either obtained from List Biological Laboratories or generously provided by Dr. Robert Munford (Department of Medicine, University of Texas, Southwestern Medical Center, Dallas, TX). Recombinant human LBP and sCD14 were obtained from XOMA Corp. (Berkeley, CA) and used interchangeably with the native proteins.

Preparation of FITC-LPS (7) and biosynthetically labeled [35S]BPI (19) was obtained from List Biological Laboratories (Campbell, CA). Metabolically labeled [3H]LPS (<1000 cpm/ng) prepared from E. coli LCD25 was either obtained from List Biological Laboratories or generously provided by Dr. Robert Munford (Department of Medicine, University of Texas, Southwestern Medical Center, Dallas, TX). Recombinant human LBP and sCD14 were obtained from XOMA Corp. (Berkeley, CA) and used interchangeably with the native proteins.

Sedimentation Analysis of LPS ± BPI—Formation of LPS-BPI complexes for sucrose density gradient analysis was accomplished by mixing [3H]LPS (100 ng/ml; 45 nm) with unlabeled BPI (1 ng/ml) (1,000 cpm) at the indicated concentrations in 0.05 M Na2HPO4/NaH2PO4, 0.14 M NaCl, 2 mM EDTA, pH 7.4 (PBSE) in 0.25 ml of total volume at 37 °C for 30 min. Aliquots (50 μl) were removed for determination of total radioactivity ([3H]LPS and [35S]BPI), and the remainder was applied to the top of a linear 5–50% sucrose gradient (4 ml) resting on a cushion of 60% sucrose (0.2 ml) and centrifuged at 18,000 RPM for 25 min in a VT-865 (Sorvall-DuPont) rotor. Fractions (13 drops) were collected and the top of a linear 5–50% sucrose gradient (4 ml) resting on a cushion of 60% sucrose (0.2 ml) and centrifuged at 18,000 RPM for 25 min in a VT-865 (Sorvall-DuPont) rotor. Fractions (13 drops) were collected and the radioactivity of FITC-LPS and [3H]LPS and [35S]BPI was determined in each fraction by liquid scintillation counting. Recoveries of [3H]LPS and [35S]BPI were >90% under all conditions.

Light Scattering of LPS Aggregates—Dispersion of LPS ± BPI or LBP were analyzed by dynamic light scattering to determine the size of LPS aggregates. A stock solution of Re595 LPS (5 mg/ml) in distilled water containing 0.5 mg/ml of human serum albumin) was sonicated at room temperature for 2 min at a power setting of 30% in a water bath sonicator (550 Sonic Disembrator; Fisher-Scientific) and diluted to 10 μg/ml (4.5 μM) in PBSE. After the addition of LBP or BPI, LPS dispersions were incubated at 37 °C for 30 min with gentle rotation and aliquots (200 μl) were filtered through a 0.1-μm Anotop 10 Plus inor-
ganic membrane filter (Whatman Inc., Clifton, NJ) to remove dust particles. The filtered solution was injected into the DYNAPRO-801 Dynamic Light Scatterer (Protein Solutions, Inc., Charlottesville, VA), and light scattering measurements were made at room temperature. At least eight measurements of each sample were carried out. The kinetics and intensity of light scattering were used to compute the translational diffusion coefficient, and from this the apparent hydrodynamic radius of the LPS aggregates, assuming a spherical (vesicular) shape as has been demonstrated for aggregates of lipid IVA (22). At the concentrations tested, LBP and BPI alone produced no detectable light scattering.

Fluorescence Assays of FITC-LPS—Fluorescence measurements using FITC-LPS were accomplished as described previously (7). To assess the ability of BPI to block FITC-LPS binding to LBP, BPI at the indicated concentrations was mixed with FITC-LPS (100 ng/ml; 45 nM) in PBSE in an albumin-coated glass cuvette in a total volume of 250 μl for 30 min at 37 °C. At time 0 on the recording, the cuvettes were put into the fluorimeter and LBP (10 μg/ml; 167 nM) was added after approximately 80 s.

RESULTS AND DISCUSSION

BPI Increases Sedimentation Velocity of LPS—Sucrose gradient analyses of [3H]LPS and [35S]BPI incubated before centrifugation, alone or together, are shown in Fig. 1A. [35S]BPI sedimented alone was recovered at the top of the gradient in fractions 12 and 13 (>70% total recovery; data not shown). [3H]LPS sedimented alone migrated a short distance into the gradient and was recovered mainly in fractions 9–11. Thus, even though monomers of LPS are of considerably smaller molecular mass than BPI (i.e. ~2300 versus 55,000 Da), the [3H]LPS sediments more rapidly than BPI, presumably reflecting aggregation of LPS. Similar results were obtained in sedimentation studies of [3H]LPS and [35S]LBP (7).

After co-incubation of LPS and BPI at 37 °C for 30 min, sedimentation of [3H]LPS increased in a manner dependent on the dose of BPI (Fig. 1A). The dose dependence was remarkable; nearly all LPS (45 nM) sedimented further than untreated LPS after preincubation with as little as 1.2 nM BPI, a ratio of approximately 40 LPS molecules per BPI molecule. Essentially all recovered [35S]BPI cosedimented with [3H]LPS (Fig. 1A), indicating that the altered sedimentation properties of LPS reflect the properties of LPS-BPI complexes. Increased sedimentation of LPS was also seen at BPI concentrations well above the LPS concentration (e.g. 120 nM BPI; LPS: BPI molar ratio, 0.37; data not shown).

In contrast, when [3H]LPS was similarly incubated with LBP at high ratios of LPS:LBP (13:1), both LBP and LPS sedimented with the mobility of uncomplexed LPS, whereas at low ratios of LPS to LBP (0.37:1) LPS and LBP co-sedimented with the velocity of LBP (7).

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mented, these findings suggest that whereas LBP disaggregates LPS under these experimental conditions, BPI either increases the density of LPS aggregates and/or cross-links aggregates of LPS into even larger aggregates.

LBP and BPI Produce Opposite Effects on the Light Scattering Properties of LPS—The contrasting effects of LBP and BPI on the physical state of LPS aggregates were further demonstrated by dynamic light scattering (Fig. 2). These experiments were carried out under the same conditions as the sedimentation studies except that 100× higher LPS concentrations (10 μg/ml; 4.5 μM) were necessary to produce detectable light scattering. Under these conditions, LPS behaved as aggregates with an apparent hydrodynamic radius of approximately 25 nm and an apparent molecular mass of approximately 7000 kDa corresponding to aggregates containing approximately 3000 LPS molecules. These properties are similar to those previously reported for aggregates of lipid IVA (22). Addition of LBP caused a dose-dependent decrease in the apparent radius of LPS aggregates, whereas BPI caused a dose-dependent increase in the apparent size of the LPS aggregates (Fig. 2). No light scattering was detected at the highest LBP concentration tested (1 μM) presumably reflecting a reduction in the size and/or concentration of LPS aggregates below the levels of detection. These findings are consistent with a disaggregating effect of LBP under these conditions, whereas BPI may stably insert into LPS aggregates to increase both the size and sedimentation rate of these particles.

Comparison of Effects of LBP and BPI on FITC-LPS—The properties of LPS-BPI complexes were explored further using FITC-LPS. We have previously observed that the fluorescence of FITC-LPS is enhanced when LBP complexes to the LPS (7), and others have observed the same phenomenon with Bodipy-LPS (8). Based on the sedimentation properties of LPS-LBP complexes, we have speculated that the increase in fluorescence was due to a reduction in fluorescein self-quenching resulting from disaggregation of LPS by LBP (7). In view of the very different sedimentation and light scattering properties of LPS complexed with LBP or BPI, the finding that BPI enhanced FITC-LPS fluorescence nearly as much as did the addition of LBP was unexpected (Fig. 3). Thus, the increased fluorescence observed upon interaction of FITC-LPS with LPS-binding proteins does not necessarily reflect LPS disaggregation. The ability of BPI to insert into lipid membranes and vesicles containing LPS² may account for its effects on LPS fluorescence by increasing the spacing between FITC-LPS molecules still residing within aggregates.

Maximum BPI-triggered enhancement of FITC-LPS fluorescence required approximately stoichiometric amounts of BPI (Fig. 4). These findings are compatible with a maximal capacity for BPI of LPS-coated surfaces of approximately 1 BPI molecule per LPS molecule (17).² Fitting of the data to a rectangular hyperbola as represented in Fig. 4 yielded an apparent $K_D$ of 3 nM. However, when a 3-fold lower concentration of FITC-LPS was used, the apparent $K_D$ also dropped by a factor of 3 (data not shown). Thus, these binding data represent a titration of available sites of FITC-LPS with BPI and therefore underesti-

² Weise, A., Brandenburg, K., Lindner, B., Schromm, A. B., Rietzchel, E. T., and Seydel, U. (1997) Biochemistry, in press.

![Figure 4](image-url)  
**Figure 4.** Dose-dependent effects of BPI on fluorescence of FITC-LPS (45 nM).

![Figure 5](image-url)  
**Figure 5.** Low concentrations of BPI inhibit fluorescence-enhancing effects of high concentrations of LBP on FITC-LPS. A, BPI at the indicated concentrations was mixed with FITC-LPS (45 nM) in PBS-E in an albumin-coated cuvette for 30 min at 37 °C. After the preincubation, the cuvettes were placed in the fluorimeter ($T_0$), LBP (170 nM) was added, and fluorescence measurements were continued until the fluorescence stabilized. B, the change in FITC-LPS fluorescence upon the addition of LBP (170 nM) as a function of the concentration of BPI in the preincubation.
mate the affinity of BPI for the LPS.

Because low concentrations of BPI can inhibit LBP-promoted LPS signaling (23–25), we investigated whether low (substoichiometric) concentrations of BPI can block the enhancing effects of LBP on the fluorescence of FITC-LPS. To this end, the fluorescence of FITC-LPS was monitored both after preincubation for 30 min with increasing amounts of BPI to permit formation of LPS-BPI complexes and after subsequent addition of LBP. The fluorescence changes observed after the addition of LBP as shown in Fig. 5A are plotted versus [BPI] in Fig. 5B. Fig. 5B shows that approximately 2 nM BPI was sufficient to block any further reaction of 45 nM LPS with 170 nM LBP. The ability of BPI to interfere with LPS-LBP interactions when present at a molar ratio of as low as 1 BPI:20 LPS suggests that the effects of BPI are due to global alterations of the LPS aggregate and not via blocking of each LPS molecule in the aggregate. Two observations are consistent with this view: 1) BPI doses that prevent LBP-triggered increased FITC-LPS fluorescence roughly correspond to the doses needed to produce the most rapidly sedimenting LPS aggregates (see Fig. 1A); and 2) the dose-dependent effects of BPI on FITC-LPS fluorescence (Fig. 4) suggest that LPS aggregates can accommodate at least one BPI per LPS, and hence not all binding sites in LPS aggregates are blocked at BPI doses that block LBP-triggered increased FITC-LPS fluorescence. We suggest that the inhibitory effect of BPI reflects alterations introduced by BPI in the physical properties of the LPS aggregate resulting either in reduced affinity of LBP for the LPS aggregate or reduced ability of LBP to remove LPS molecules from these aggregates.

In summary, this study documents for the first time marked differences in the physical properties of LPS-BPI versus LPS-LBP complexes. Release of individual LPS molecules from bacterial membranes or cell-free aggregates is generally believed to be important for optimal LPS signaling (3, 4). Thus, the ability of LBP and/or CD14 but not of BPI to induce disaggregation of LPS as judged from sedimentation and light scattering analyses (Refs. 7 and 8; Figs. 1A and 2) is in line with the stimulatory and inhibitory effects on LPS signaling of LBP and BPI, respectively. Further, the potent LPS neutralizing activity of BPI in body fluids where LBP is also present is consistent with the demonstration that BPI has a much higher affinity for LPS than LBP (26) and with the ability of BPI, even at substoichiometric levels, to block LPS interactions with LBP. Future studies making use of BPI-LBP chimeras and conditions that allow bioassay of LPS activity should permit a better definition of the structural basis of the differences in the physical properties of LPS complexes with BPI and LBP and the importance of these differences in the opposing functional properties of these two closely related LPS-binding proteins.

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