Catalytic Properties of Lipopolysaccharide (LPS) Binding Protein

TRANSFER OF LPS TO SOLUBLE CD14*

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Lipopolysaccharide (LPS) binding protein (LBP) is a lipid transfer protein that catalyzes transfer of LPS monomers from micelles to a binding site on soluble CD14 (sCD14) and transfer of LPS from LPS-sCD14 complexes to HDL particles. To characterize the first of these two reactions, LPS covalently derivatized with the fluorophore, boron dipyrromethene difluoride (BODIPY), was used to monitor LBP-catalyzed movement of LPS in real time. The fluorescence efficiency of micelles of BODIPY-LPS was low but was strongly increased upon dissolution in detergent or upon binding to sCD14. Spontaneous binding of BODIPY-LPS to sCD14 was very slow but was accelerated by substoichiometric concentration of LBP, and the rate of binding was measured under a variety of conditions. LBP-catalyzed transfer was first order with respect to both sCD14 and LPS concentration, and the apparent Michaelis constant of LBP, and the rate of binding was measured under a variety of conditions. LBP-catalyzed transfer was first order with respect to both sCD14 and LPS concentration, and the apparent Km values were 1–2 μg/ml for sCD14 and 100 ng/ml for LPS. The maximum turnover number for LBP was approximately 150 molecules of LPS min⁻¹ LBP⁻¹. LBP alone caused a small but measurable increase in the fluorescence of BODIPY-LPS, suggesting that it bound LPS aggregates but did not readily remove LPS monomers. The subsequent addition of sCD14 caused a large fluorescence increase, suggesting transfer of BODIPY-LPS to sCD14. These and other observations suggest that LPS is transferred by an ordered ternary complex reaction mechanism in which LBP transfers LPS monomer from LPS aggregates to sCD14 without dissociating from the LPS aggregate.

Mammals mount an innate immune response to Gram-negative bacteria by recognizing lipopolysaccharide (LPS, endotoxin), an amphipathic molecule that forms micelles in aqueous buffers. Micelles of LPS bind poorly to leukocytes and provoke a response only at very high concentrations. The addition of buffers. Micelles of LPS bind poorly to leukocytes and provoke a response only at very high concentrations. The addition of these two reactions, LPS covalently derivatized with the fluorophore, boron dipyrromethene difluoride (BODIPY), was used to monitor LBP-catalyzed movement of LPS in real time. The fluorescence efficiency of micelles of BODIPY-LPS was low but was strongly increased upon dissolution in detergent or upon binding to sCD14. Spontaneous binding of BODIPY-LPS to sCD14 was very slow but was accelerated by substoichiometric concentration of LBP, and the rate of binding was measured under a variety of conditions. LBP-catalyzed transfer was first order with respect to both sCD14 and LPS concentration, and the apparent Km values were 1–2 μg/ml for sCD14 and 100 ng/ml for LPS. The maximum turnover number for LBP was approximately 150 molecules of LPS min⁻¹ LBP⁻¹. LBP alone caused a small but measurable increase in the fluorescence of BODIPY-LPS, suggesting that it bound LPS aggregates but did not readily remove LPS monomers. The subsequent addition of sCD14 caused a large fluorescence increase, suggesting transfer of BODIPY-LPS to sCD14. These and other observations suggest that LPS is transferred by an ordered ternary complex reaction mechanism in which LBP transfers LPS monomer from LPS aggregates to sCD14 without dissociating from the LPS aggregate.

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The abbreviations used are: LPS, lipopolysaccharide; BODIPY, boron dipyrromethene difluoride; BODIPY-LPS, BODIPY-labeled LPS; LBP, LPS binding protein; PD, Dulbecco’s phosphate-buffered saline lacking Ca²⁺ and Mg²⁺; PBS, Dulbecco’s phosphate-buffered saline with Ca²⁺ and Mg²⁺; sCD14, soluble CD14.
caused by LBP might also result from a small fraction of the large complexes in solution (14, 15). The fluorescence increase with recent work showing that LBP and LPS may form very large complexes in solution (5) and also consistent with our finding that radioactive LPS could not be found associated with monomeric LBP in native gels (5) and results in maximal upon addition of only 0.2 mol of LBP/mol of LPS. It is 75% of the observation that fluorescence enhancement is 75% of the maximal change in the emission spectrum with a strong maximum at 518 nm (Fig. 1B). This observation indicates that the BODIPY in our preparation is aggregated, most likely because it is coupled to LPS, and the BODIPY-LPS exists in micelles or aggregates. Assuming that BODIPY-LPS behaves similarly to BODIPY-ceramide, the ratio of fluorescence intensity at 620 and 515 nm suggests that our BODIPY-LPS preparation contained ~20 mol % BODIPY. This estimate is consistent with our measurement of 0.2 mol of BODIPY/mol of LPS (see “Materials and Methods”).

Dissolution of BODIPY-LPS micelles with SDS led to a pronounced change in the emission spectrum with a strong maximum at 518 nm (Fig. 2A). This observation confirms the attachment of BODIPY to LPS and indicates that fluorescent properties report the aggregation state of BODIPY-LPS. Since the emission at 518 nm rises up to 50-fold upon disaggregation of BODIPY-LPS, we have used fluorescence intensity at this wavelength as a measure of disaggregation.

BODIPY-LPS does not effectively disaggregate LPS—BODIPY is known to bind immobilized LPS and stably coat the surface of LPS-coated plastic plates (11, 12), LPS-coated erythrocytes or whole Gram-negative bacteria (13). To determine if binding of LBP changes the aggregation state of LPS in solution, BODIPY-LPS aggregates were mixed with increasing concentrations of LBP, and fluorescence emission at 518 nm was measured. LBP caused a reproducible, dose-dependent increase in fluorescence, but even a molar excess of LBP caused fluorescence to rise only 2-fold (Fig. 2). This slight change in fluorescence may be caused by the interaction of LBP with BODIPY-LPS that remains aggregated in micelles. This hypothesis is consistent with the observation that fluorescence enhancement is 75% of maximal upon addition of only 0.2 mol of LBP/mol of LPS. It is also consistent with our finding that radioactive LPS could not be found associated with monomeric LBP in native gels (5) and with recent work showing that LBP and LPS may form very large complexes in solution (14, 15). The fluorescence increase caused by LBP might also result from a small fraction of the BODIPY-LPS becoming associated with soluble, monomeric LBP, and our data cannot determine whether LBP exerts its effects on fluorescence by binding to BODIPY-LPS micelles or by disassociating a small proportion of BODIPY-LPS from micelles. In either case, it is clear that LBP does not cause substantial disaggregation of LPS.

RESULTS

The Fluorescence Spectrum of BODIPY-LPS Reports Its Aggregation State—BODIPY is an efficient, photostable fluorescent probe whose emission spectrum depends on the local concentration of fluorophore. For example, vesicles containing 50 mol % of BODIPY-ceramide have an emission maximum at 620 nm, but dilution of the BODIPY-ceramide to 2 mol % with phosphatidylcholine shifts the emission maximum to ~515 nm. The ratio of fluorescence intensity at 515–620 nm changes from 0.01 to 10 upon dilution in this way (10). The emission spectrum of BODIPY-LPS in PD (Fig. 1A) showed a broad peak at 620 nm, indicative of a high local concentration of fluorophore. This observation indicates that the BODIPY in our preparations is aggregated, most likely because it is coupled to LPS, and the BODIPY-LPS exists in micelles or aggregates. Assuming that BODIPY-LPS behaves similarly to BODIPY-ceramide, the ratio of fluorescence intensity at 620 and 515 nm suggests that our BODIPY-LPS preparation contained ~20 mol % BODIPY. This estimate is consistent with our measurement of 0.2 mol of BODIPY/mol of LPS (see “Materials and Methods”). Dissolution of BODIPY-LPS micelles with SDS led to a pronounced change in the emission spectrum with a strong maximum at 518 nm (Fig. 1B). This observation confirms the attachment of BODIPY to LPS and indicates that fluorescent properties report the aggregation state of BODIPY-LPS. Since the emission at 518 nm rises up to 50-fold upon disaggregation of BODIPY-LPS, we have used fluorescence intensity at this wavelength as a measure of disaggregation.

FIG. 1. Emission spectrum of BODIPY-LPS. The fluorescence spectrum of 10 μg/ml BODIPY-LPS, with (B) or without (A) 2% SDS was recorded at room temperature with excitation at 485 nm. Note the different scales on the y axis.

FIG. 2. Fluorescence change caused by LBP/BODIPY-LPS interaction. 16.5 nM (50 ng/ml) BODIPY-LPS was mixed with increasing concentrations of LBP in PD buffer at 37 °C. The fluorescence at 518 nm rose rapidly (not shown) and was measured at the plateau (about 20 s after the addition of LBP). The data are the mean of three fluorescence measurements ± S.D. of a representative experiment repeated three times. Fluorescence of 16.5 nM BODIPY-LPS in 2% SDS was 0.352.
BODIPY-LPS was incubated with 1 ng/ml LBP and 200 ng/ml sCD14 in different combinations to determine the fluorescence intensity of BODIPY-LPS in the presence of low amounts of LBP to facilitate binding (see below), caused a strong rise in fluorescence (Fig. 3, line A), which reached a plateau at about 10 min. The maximal fluorescence reached at plateau was dependent on the concentration of sCD14. Substoichiometric amounts of sCD14 caused a concentration-dependent, linear increase in fluorescence that reached a maximum at approximately 1 CD14 added per BODIPY-LPS molecule (Fig. 4). This finding indicates that BODIPY-LPS binds sCD14 and suggests that the stoichiometry of this interaction is approximately one LPS per sCD14, consistent with our previous findings (5). Parallel measurements, however, showed that addition of SDS to BODIPY-LPS caused a fluorescence increase even greater than that observed with saturating amounts of sCD14. Two phenomena, individually or in combination, could contribute to this observation: the fluorescence increase upon binding sCD14 could be less than that caused by dissolution in SDS micelles, or a portion of BODIPY-LPS may have failed to bind to sCD14 and remained as aggregates. Experiments described below show that the fluorescence efficiency of BODIPY-LPS/sCD14 complexes is less than that of BODIPY-LPS in SDS micelles.

To obtain a preparation of BODIPY-LPS/complexes that did not contain any free BODIPY-LPS micelles, 200 ng/ml BODIPY-LPS was incubated with 1 μg/ml LBP and a 15-fold molar excess of sCD14 (50 μg/ml) for longer than 300 s to achieve maximal transfer. In the presence of sCD14 and LBP, the fluorescence intensity at 518 nm rose 10.5-fold (from 0.031 to 0.322). The addition of SDS to this mixture caused the fluorescence to rise a further 2-fold, confirming that sCD14 had not fully increased the fluorescence efficiency. The mixture was then passed through an ultrafiltration membrane with a 100-kDa size cutoff (see “Materials and Methods”). Previous studies showed that both sCD14 and LPS/sCD14 complexes pass through the membrane, but neither LPS micelles alone nor LBP pass this filter (6). We verified that less than 2.5% of BODIPY-LPS alone passed through the filter (not shown). The ultrafiltrate showed strong fluorescence at 518 nm with an emission spectrum identical to that of BODIPY-LPS in SDS (not shown). This observation confirms that BODIPY-LPS/sCD14 complexes are formed and that they show the fluorescence properties of disaggregated fluorophore. The addition of SDS directly to the ultrafiltered complexes caused the fluorescence to rise. After correction for background, the fluorescence of BODIPY-LPS/sCD14 complexes was 41.3 ± 2.3% (n = 3) of BODIPY-LPS in 2% SDS. This observation indicates that BODIPY-LPS in complex with sCD14 shows lower fluorescence efficiency than BODIPY-LPS in SDS, and we have used this finding to calculate the concentration of BODIPY-LPS/sCD14 complexes. On the day of each experiment, we measured the fluorescence of BODIPY-LPS alone and in the presence of 2% SDS. A curve relating fluorescence to BODIPY-LPS/sCD14 concentration was then plotted with 0% as the fluorescence value of BODIPY-LPS alone and 100% as the fluorescence value of BODIPY-LPS measured in SDS × 0.41. We have used this method to determine the amount of BODIPY-LPS bound to sCD14 in the presence of excess BODIPY-LPS (Fig. 4, left y axis). At several sCD14 concentrations, a constant proportion of 1 BODIPY-LPS appeared bound per sCD14.

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To verify that the movement of BODIPY-LPS into sCD14 was caused by LBP rather than a contaminant or buffer constituent, anti-LBP IgG was added to the reaction. Rabbit IgG was caused by LBP rather than a contaminant or buffer component. Stoichiometric levels, its function appears to be catalytic.

binding site are deleted (sCD14D57–64). This mutant sCD14 shows strongly decreased ability to bind LPS in gel assays or to mediate cellular responses to LPS (9), and we found that sCD14D57–64 showed a strongly decreased ability to enhance fluorescence of BODIPY-LPS under the same experimental conditions (Fig. 6).

In the above studies, sCD14 was present in stoichiometric excess of LBP. In blood, however, approximately equal levels of LBP and sCD14 are present, and Tobias et al. (15) have suggested that under these conditions the LPS will remain with LBP and will not be transferred to sCD14. We tested this hypothesis by measuring the interaction of BODIPY-LPS with equimolar LBP and sCD14 (Fig. 7). LBP alone, at a concentration stoichiometric to LPS (line B), caused some enhancement of the fluorescence of BODIPY-LPS. When stoichiometric sCD14 was added to the mixture of LBP and BODIPY-LPS the fluorescence increased very rapidly (line D), suggesting rapid transfer to sCD14. The level of fluorescence obtained was similar to that seen with substoichiometric LBP (line E). This result suggests that BODIPY-LPS is substantially transferred to sCD14 even in the presence of stoichiometric levels of LBP.

Catalytic Constants for LBP-mediated Transfer of BODIPY-LPS to sCD14—The initial rate for transfer of BODIPY-LPS to sCD14 was obtained over a range of concentrations of both sCD14 and BODIPY-LPS (Fig. 8). The transfer reaction was first order with respect to concentration of both sCD14 and BODIPY-LPS (not shown). Measurement performed in buffer PD (without added divalents) yielded a Kₘ (apparent) for LPS of 88.8 ± 11 ng/ml (n = 2) with 1 μg/ml sCD14 and 133.8 ± 1.6 ng/ml (n = 2) with 5 μg/ml sCD14. The Kₘ (apparent) for sCD14 was 1.7 ± 0.5 μg/ml (n = 2) with 30 ng/ml LPS and 1.5 ± 0.8 μg/ml (n = 2) with 60 ng/ml LPS. To determine if these values are sensitive to the concentration of divalent cations, additional measurements were made in PBS (a buffer with physiologic concentration of Ca²⁺ and Mg²⁺). This addition caused very slight changes; we observed a Kₘ (apparent) for LPS of 91.1 ± 13.0 ng/ml (n = 2) with 1 μg/ml sCD14 and 165.0 ± 42 ng/ml (n = 2) with 5 μg/ml sCD14. The Kₘ (apparent) for CD14 was 0.91 ± 0.5 (n = 2) with 30 ng/ml LPS and 1.47 ± 1.47 (n = 2) with 60 ng/ml LPS. The turnover number for LBP was 59.3 ± 16 mol of LPS min⁻¹ LBP⁻¹ with 5 μg/ml sCD14, and an intercept replot of Fig. 8A (not shown) yielded an extrapolated maximum turnover number of 149 ± 22 mol of LPS min⁻¹ LBP⁻¹.

DISCUSSION

Here we describe a sensitive method for measuring the movement of labeled LPS from micelles to sCD14. The method

![Image](https://example.com/image1.png)

**Fig. 5.** Anti-LBP antibody blocks transfer activity of LBP. Anti-LBP (line B) or nonimmune rabbit IgG (line A) (20 μg/ml) was incubated with LBP (40 ng/ml) for 5 min at room temperature. BODIPY-LPS (50 ng/ml) and sCD14 (2 μg/ml) were then added, and the fluorescence of the mixtures was recorded over time at 30°C. The other lines are the mixtures as indicated in the graph. The experiment is representative of three repeats.

![Image](https://example.com/image2.png)

**Fig. 6.** sCD14D57–64 fails to enhance the fluorescence of BODIPY-LPS. 13.2 nm BODIPY-LPS and 0.67 nm LBP were mixed with either 5 μg/ml full-length sCD14 (line A) or sCD14D57–64 (line B), and fluorescence at 518 nm was recorded over time at 30°C. Control mixtures were as shown on the graph. The figure is representative of two separate experiments.

![Image](https://example.com/image3.png)

**Fig. 7.** BODIPY-LPS is transferred to sCD14 in the presence of equimolar LBP. In lines A–C, BODIPY-LPS (33 nm), LBP (33 nm), and sCD14 (36 nm) were mixed as shown, and fluorescence was recorded for 500 s at 30°C. In line D, BODIPY-LPS and LBP were mixed and measured for 200 s. sCD14 was then added, and measurement was continued. In line E, 10-fold less LBP (33 nm) was employed. Results are representative of three repeats.
depends on a spectral shift in a fluorophore upon dilution and is similar in principal to a method recently described by Tobias et al. (15). Dilution of our BODIPY-LPS yields up to a 50-fold rise in fluorescence. Like Tobias et al., we confirm the finding of Hailman et al. (5) that LBP catalyzes transfer of LPS from micelles to sCD14 and that sCD14 binds approximately one LPS molecule. We further show that the transfer exhibits first order kinetics and we have defined the catalytic constants for the transfer reaction. We find that the $K_m$, for sCD14 is 1-2 $\mu$g/ml, values within the range of concentrations of sCD14 in human plasma. The $K_m$ for LPS (~100 ng/ml), on the other hand, is substantially higher than the LPS levels normally observed in sepsis, and LBP may thus normally operate with levels of this substrate well below its $K_m$. With saturating levels of LPS and sCD14, the turnover number for LBP is approximately 150 mol of LPS min$^{-1}$ LBP$^{-1}$.

Catalytic Reaction Mechanism for Transfer of LPS to sCD14—As with bisubstrate enzymes (16), two general models may explain the interaction of the catalyst, LBP, with the substrates, sCD14 and LPS micelles. In a “ping pong” or “binary complex” model, LBP interacts with an LPS micelle in a first bimolecular reaction. LBP then dissociates from the micelle with one molecule of LPS and binds to sCD14 in a second bimolecular reaction (Fig. 9A). Alternatively, in a “ternary complex” model, LBP interacts simultaneously with both an LPS micelle and sCD14 during transfer of an LPS monomer to the sCD14 (Fig. 9B). We have drawn this as an ordered ternary complex reaction with LBP binding to LPS micelles before sCD14 since LBP is known to bind LPS aggregates without sCD14 (11, 12), but sCD14 does not bind LBP without LPS (13). Several lines of evidence indicate that LBP utilizes principally the ternary complex intermediate to transfer LPS to sCD14. 1) The binary complex model predicts parallel lines in the double reciprocal plots of Fig. 8 (16). These plots, however, clearly intersect, suggesting a ternary complex mechanism. A secondary plot of Fig. 8A, shown in the inset, confirms a monotonic change in slope with CD14 concentration. 2) A ternary complex of aggregated LPS, LBP, and sCD14 clearly does form. Erythrocytes coated with LPS-LBP complexes bind to CD14 on the surface of macrophages (13), complexes of LBP and LPS are removed from plasma upon chromatography on immobilized CD14 (17), and complexes of labeled LBP and LPS bind to CD14-bearing cells in a CD14-dependent manner (14, 18). 3) The binary complex model requires that LBP dissociate from LPS micelles to yield an LBP-LPS complex that shuttles LPS through the aqueous medium. This intermediate, however, has not been demonstrated under physiologic conditions. We have failed to observe monomeric LPS-LBP complexes in native gels of mixtures of LPS and LBP (5). Moreover, LBP binds with high affinity to LPS-coated surfaces (11, 12), suggesting that disassociation is energetically unfavorable. The ternary complex model obviates the need for this unfavorable reaction. While Tobias et al. (15) have observed monomeric LBP complexed with one to two LPS molecules under certain conditions, these studies were done in the presence of EDTA, an agent known to destabilize LPS. We have observed that EDTA caused a marked enhancement of the fluorescence of LBP-BODIPY-LPS complexes (not shown), suggesting that it could promote disaggregation. Our studies were done in the absence of EDTA to avoid that artificial disaggregation. Additional studies documented that the addition of physiologic concentration Ca$^{2+}$ and Mg$^{2+}$ did not significantly affect reaction kinetics. 4) Shuttling of LPS in a complex with LBP is required for the binary complex mechanism but is inconsistent with other data on the action of LBP. LBP is known to mediate the three transfer reactions in Fig. 10. If LPS-LBP complexes were the intermediate in these reactions, the addition of sCD14 would not affect the rate of transfer of LPS to HDL; sCD14 could not generate LPS-LBP complexes in excess of the
LBP complexes needed to make LPS-sCD14. We have observed, however, that the addition of sCD14 dramatically enhances movement of LPS from micelles to HDL (6). This finding is inconsistent with the binary complex model of LBP action but is consistent with a model in which transfer of LPS monomers to sCD14 is catalyzed through specific interactions of LBP with both the LPS micelles and sCD14. We wish to emphasize that our data do not rule out the possibility of some LPS being shuttled by LBP in a binary complex reaction. They merely suggest that a ternary complex mechanism is likely to be favored under physiological conditions.

Our conclusion that LBP transfers LPS via an ordered, ternary complex reaction model differs from that of Tobias et al. (15), who favor the binary reaction model of Fig. 9A. These authors did not address the possibility of the ternary complex reaction of Fig. 9B. They argued that, since LBP binds to LPS with high apparent affinity, transfer to sCD14 would be energetically unfavorable. Our results indicate that, even with equimolar LBP and sCD14, transfer of LPS to sCD14 is favored (Fig. 7). How could LBP transfer LPS to sCD14 if its affinity for LPS is greater than that of CD14? We suggest that the high affinity of LBP is for the LPS aggregate, not the LPS monomer. A key feature of the ternary complex reaction model is that LBP may transfer an LPS monomer from an aggregate without the energetically unfavorable step of disassociating from the aggregate. One LBP molecule may thus transfer successive LPS monomers from a single aggregate (see curved arrow in Fig. 9B). This formulation is consistent with all known observations on the action of LBP.

While our results strongly suggest an ordered ternary complex mechanism for LBP-mediated transfer of LPS from micelles to sCD14, they do not describe the reaction mechanism for the other two reactions catalyzed by LBP: transfer of LPS from micelles to HDL particles and transfer of LPS monomers from sCD14 to HDL particles (see Fig. 10). Experiments to describe these reactions are currently under way.

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Fig. 10. Three transfer reactions facilitated by LBP: LBP catalyzes movement of LPS monomer from LPS aggregates to sCD14 (5), from LPS aggregates to HDL particles (7), and from LPS-sCD14 complexes to HDL particles (6).
