Fluorescence Resonance Energy Transfer Analysis of Lipopolysaccharide in Detergent Micelles

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ABSTRACT Bacterial endotoxins or lipopolysaccharides (LPS), cell wall components of gram-negative bacteria, are involved in septic shock. LPS consists of a lipid A tail attached to core and O-antigen polysaccharides, but little is known about the supramolecular structure of LPS in blood. We have developed an approach to locate donor and acceptor probes in sulfobetaine palmitate detergent micelles using steady-state and time-resolved fluorescence resonance energy transfer. C18-fluorescein and several LPS species of varying molecular weight labeled with fluorescein isothiocyanate (FITC-LPS) were the donor probes. Acceptor probes were 1,1-dilinoleyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (Fast C18-DiI, $R_0 \approx 68$ Å), and octadecyl B rhodamine chloride (C18-Rhd, $R_0 \approx 58$ Å). With either acceptor, the transfer was of similar high efficiency when FITC-LPS *Salmonella minnesota* Re 595 (2,500 mol wt, lacking both core and O-antigen) or C18-fluorescein were the fluorescent donor probes. Thus, the donor FITC-LPS with short polysaccharide chain *S. minnesota* Re 595 and the control donor C18-fluorescein appear to be close to the micelle surface. The transfer efficiency decreased as the molecular weight of the LPS increased. Separation distances between the longest FITC-LPS, *S. minnesota* (20,000 mol wt, with a long O-antigen), and the micelle were estimated to be 1.5 R_0 or more (~100 Å), consistent with an extended conformation for the longer O-antigen polysaccharide chain in the detergent.

INTRODUCTION

Bacterial endotoxins or lipopolysaccharides (LPS) are a cell wall product from gram-negative bacteria that can initiate septic shock in animals and humans (Raetz, 1990). During a gram-negative infection LPS is shed from the bacterial outer wall into the bloodstream either as single molecules or as cell wall fragments. LPS complexes with a serum lipopolysaccharide-binding protein, LBP (Tobias et al., 1989). The LPS-LBP complex can interact with membrane-bound CD14 on myeloid cells or with soluble CD14 in plasma. LPS binding to CD14 on monocytes leads to monocyte activation and secretion of tumor necrosis factor and interleukin-1 (Wright et al., 1990; Lee et al., 1993). LPS bound to soluble CD14 activates epithelial and endothelial cells to express surface adhesion molecules and secrete cytokines (Pugin et al., 1993).

LPS is composed of three parts (Fig. 1), a negatively charged lipid A with six or seven fatty acid chains linked to a nonreducing, biphosphorylated, glucosamine disaccharide head group, which anchors the LPS to the outer monoleaflet of the bacterial outer membrane; a core oligosaccharide covalently bound to the lipid A moiety via 3-deoxy-D-manno-octulosonic acid; and the O-antigen polysaccharide chain, which protrudes into the surrounding medium (Lugtenberg and Van Alphen, 1983; Nikaido and Vaara, 1985; Raetz, 1990; Rietschel et al., 1994). Sites of attachment of 4-amino-4-deoxy-L-arabinose and phosphoethanolamine to the lipid A molecule are nonstoichiometric and not well characterized (Raetz, 1990). Lipid A subunits from different bacterial strains have similar chemical compositions, and synthetic lipid A has been shown to retain endotoxicity (Lüderitz et al., 1989). LBP has been shown to interact with the negatively charged lipid A portion of the LPS (Tobias et al., 1989). The carbohydrate cores and the O-antigen chain vary both in composition and in the number of polysaccharide repeat units, but the mechanisms for the differing toxicities between bacterial strains have not been fully resolved. The lipid A moiety of the LPS molecule has been shown to be responsible for the endotoxic effect on cells (Brade et al., 1987). Research has progressed on LPS immunobiology (Skelly et al., 1979; Brade et al., 1987; Schumann et al., 1990; Raetz et al., 1991; Gallay et al., 1993; Pugin et al., 1993) whereas the organization and structure of LPS aggregates in the blood and their involvement in the onset of septic shock is still not well understood.

Chemical and biophysical methods have been used to study short-chain LPS mutants and lipid A (Van Alphen et al., 1980; Qureshi et al., 1985; Labischinski et al., 1985; Seydel et al., 1989; Brandenburg et al., 1990, 1992, 1993; Holst et al., 1993; Kastowsky et al., 1990, 1993). It has been suggested that the lipid A activity is modulated by the oligo- or polysaccharide chain (Lüderitz et al., 1989; Yeh and Jacobs, 1992) and that LPS may aggregate into different structures, including micelles, inverted micelles, or bilayers, and undergo lamellar to inverted hexagonal or cubic phase transitions in vitro (Brandenburg and Seydel, 1984; Bran-

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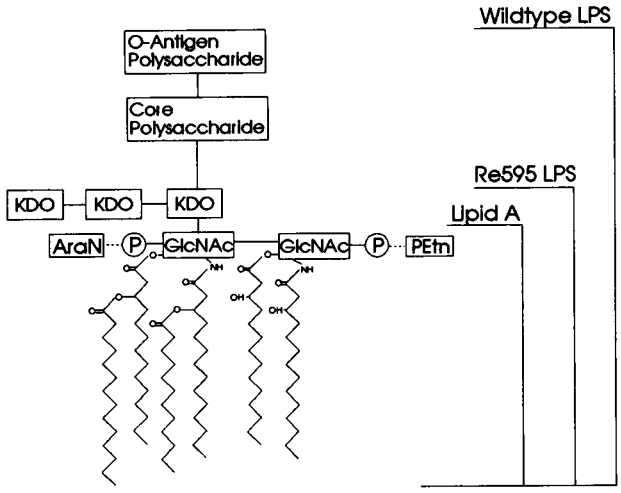

FIGURE 1 Schematic of LPS structure. For details, see the text.

denburg, 1993). Although labeling LPS with fluorescein isothiocyanate (FITC) has permitted the analysis of the interaction of labeled LPS with target cells and binding proteins (Tobias et al., 1995; Gegner et al., 1995), the FITC-LPS aggregates with fluorescence quenching as a consequence. The aggregation obscures analysis of the LPS conformation and quantification of LPS-LBP-CD14 interactions in vitro.

Our goal in this work was to define a system for characterizing the organization of LPS monomers. We chose a detergent with low critical micelle concentration (CMC) suitable for fluorescence resonance energy transfer (FRET) (Förster, 1948). FRET has already been shown to provide insight into several aspects of lipid organization including surface density (Fung and Stryer, 1978), placement of fluorescent lipid analogues on or in lipid particles (Sklar et al., 1980), and distances between membranes and protein complexes (Holowka and Baird, 1983; Remmers and Neubig, 1993; Valenzuela et al., 1994). We have used several methods to characterize the interactions of the probe species with the detergent micelles and FRET to define, semiquantitatively, the distances between donor-labeled LPS or lipid donors and lipid acceptors. Even though the LPS species are potentially heterogeneous in size and site of conjugation, the limited energy transfer that has been observed implies distances of 100 Å or more and an extended conformation of a large fraction of the FITC-LPS polysaccharide chains from wild-type bacterial strains.

MATERIALS AND METHODS

Materials

The following chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO): FITC-labeled bacterial LPS (*Salmonella minnesota, S. typhimurium, S. abortus equi, Serratia marcescens, Escherichia coli* 0111:B4), unlabeled *S. minnesota* Re 595 LPS, *N*-phenyl-1-naphthylamine (NPN), TRIS-preset crystals (2-amino-2-(hydroxymethyl-1,3-propanediol and TRIS-hydrochloride), sodium chloride, sulfobetaine palmitate (SBP; *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propane sulfonate). Fast 1,1-dilinoleyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (C18-DiI), octadecyl rhodamine B, chloride salt (C18-Rhd), fluorescein, FITC, fluorescein octadecyl ester (C18-fluorescein), and 2- and 12-(9-anthroyloxy)-stearic acids were obtained from Molecular Probes (Eugene, OR).

Sample preparation

The freeze-dried FITC-LPS from Sigma Chemical Co. and *S. minnesota* Re595 were labeled according to Skelly et al. (1979). All FITC-LPS were rehydrated in a buffer containing 0.02 M TRIS and 0.15 M NaCl at pH 7.5 and used without further purification. The buffer was filtered through a Supor-200 (Gelman Sciences, Ann Arbor, MI), 0.2-μm filter and degassed before use. A stock solution of the detergent, 0.1 M SBP, was prepared in distilled water. In all experiments except where noted we used a concentration of 100 μM SBP in buffer. The lyophilized fluorescent acceptor probes Fast C18-DiI, C18-Rhd, and C18-fluorescein were dissolved into 100% ethanol stock solutions of 10 mg/ml; fluorescein was dissolved at 1 mM. All solutions were stored at −20°C when not in use. In the FRET experiments, small aliquots of acceptor probe were titrated into the FITC-LPS/SBP mixture. Experiments on the different strains of FITC-LPS were performed at a concentration of 30 ng/ml.

Surfactant CMC determination

The CMC of the SBP was determined according to Brito and Vaz (1986) in a buffer containing 0.02 M TRIS, 0.15 M NaCl at pH 7.5. As the NPN partitions into the hydrophobic compartment of the micelle, its emission peak shifts from 460 to 425 nm and the quantum yield increases. The increase in fluorescence was measured for 5 μM NPN (SBP varying from 0 to 200 μM). The samples were incubated for 30 min at 25°C. Fluorescence was measured (excitation 350 nm, emission 425 nm) in a Spex Fluorolog (Spex Industries, Edison, NJ) with 1.7-nm resolution.

LPS in SBP micelles

The low CMC of SBP makes it possible to accommodate a high surface density of acceptor probes at low acceptor concentration, limiting optical artifacts (inner and outer filter effects) during the measurements. The ratio of FITC molecules per LPS molecule varies in different bacterial strains because of the heterogeneity of polysaccharide repeat units in the LPS structure (mutant or wild-type strains), the actual conjugation site of the FITC, and the number of conjugation sites per LPS molecule. The ratio of FITC to LPS molecules was calculated to range from 1:4 to 1:6 (based on the FITC/LPS molecular weight ratio). The ratio of LPS molecules to micelles was on the order of 1 LPS per 20–200 micelles, depending upon the size of the LPS. Acceptor molecules were titrated into the system, reaching a maximum of two to three acceptors per micelle. At a density of one acceptor/micelle, the optical density of DiI (540 nm) or C18-Rhd (550 nm) is ~0.05. Because increasing SBP greater than 100 μM does not increase donor or acceptor fluorescence, we conclude that they are randomly distributed and quantitatively inserted into the micelles.

LPS monomer size distribution

The apparent molecular weight and size distribution of FITC-LPS was examined by one-dimensional polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970), modified with solubilization in deoxycholate (n_{agg} = 5–19 monomers per micelle) for LPS by Komuro and Galanos (1988). Low molecular weight electrophoresis standards were obtained from Bio-Rad, Richmond, CA. The deoxycholate-PAGE gel was examined for fluorescence-stained FITC-LPS polysaccharide bands by a fluorescent lamp before staining with silver (Tsai and Frasch, 1982).

Determination of free FITC in LPS

To quantify non-LPS-associated FITC label in the different LPS preparations we measured the fluorescence at 520 nm in the supernatant of LPS preparations before and after precipitation by calcium chloride (50 mM). FITC-LPS (10 mg/ml) was incubated on ice for 30 min in phosphate-buffered saline buffer, containing 2.4 mM NaH_2PO_4, 7.6 mM Na_2HPO_4, 0.145 M NaCl at pH 7.5. The precipitated FITC-LPS was pelleted in a microcentrifuge at 10,000 rpm for 20 min and fluorescence was recorded from the supernatant.

Absorbance spectroscopy

The extinction coefficients for the probes were determined in an Hitachi L-3515 spectrophotometer. In methanol, the values used were as follows: C18-DiI (549 nm) = 150,000 M^{-1} cm^{-1}; C18-fluorescein (504 nm) = 95,000 M^{-1} cm^{-1}; C18-Rhd (556 nm) = 93,000 M^{-1} cm^{-1}; and FITC (495 nm) = 76,000 M^{-1} cm^{-1} (Haugland, 1992). In SBP, the values determined were as follows: C18-DiI (550 nm) = 116,000 M^{-1} cm^{-1}; C18-fluorescein (518 nm) = 85,000 M^{-1} cm^{-1}; FITC-LPS *S. minnesota* (490 nm) = 67,900 M^{-1} cm^{-1}; and C18-Rhd (560 nm) = 62,400 M^{-1} cm^{-1}.

Fluorescence spectroscopy

Quenching of donor and acceptor probe molecules inserted in the micelle was examined by Stern-Volmer analysis in the presence of potassium iodide. FRET measurements were performed at 25°C in a Spex Fluorolog 112-1680 with a Hamamatsu R928 photomultiplier tube. Disposable acrylic cuvettes (VWR Scientific, New Plainfield, NJ) with 1-cm path lengths were used for all experiments. To minimize direct acceptor excitation, the FITC-LPS samples were excited at 470 nm. Steady-state intensity measurements of samples being analyzed for fluorescence lifetimes (below) and steady-state polarization measurements, were performed in an SLM Aminco 8000 (SLM Instruments, Rochester, NY). Excitation was 490 nm using a 490 interference filter (10-nm bandpass; Corion, Hollister, MA). Emission was measured through a 3–70 long-pass filter (Kopp, Pittsburgh, PA) in combination with a 520-nm interference filter (Corion).

Time-resolved analysis of FRET

Fluorescence lifetimes were measured in an SLM 4850 MHF spectrofluorometer (SLM Instruments) operated at 4–200 MHz. Excitation was provided by an argon ion laser at 488 nm. FITC-LPS (300 ng/ml) was detected through a Kopp 3–70 long-pass filter and a 520 (10-nm bandpass) interference filter (Corion). Experiments were performed at 25°C in glass cuvettes with 1-cm path lengths. The reference was a suspension of oyster glycogen. Data analysis was performed using a nonlinear least squares routine for fitting experimental lifetimes supplied by the manufacturer. Goodness-of-fit calculations used the default values of 0.5° and 0.5% as the uncertainties in the measured phase angle and modulation amplitude, respectively.

THEORY AND CALCULATIONS

Fluorescence resonance energy transfer

The transfer efficiency T is given by

$$T = 1 - F_a/F_o\,, \tag{1}$$

where F_o refers to the unquenched fluorescence intensity of the donor and F_a refers to the fluorescence intensity of the donor in the presence of a micelle-partitioned acceptor probe. The distance of 50% transfer, R_o, (Förster, 1948) is

$$R_o = (Q_o J \kappa^2 n^{-4})^{1/6} \times 9.79 \times 10^3 \text{Å}\,, \tag{2}$$

where Q_o is the quantum yield of the donor in the absence of acceptor, n is the index of refraction of the medium (for H_2O, $n = 1.33$), κ^2 is the dipole-dipole orientation factor (see below), and J is the overlap integral:

$$J = \int F(\lambda)\epsilon(\lambda)\lambda^4 d\lambda \Big/ \int F(\lambda) d\lambda\,, \tag{3}$$

where $\epsilon(\lambda)$ is the extinction coefficient of the acceptor probe (M^{-1} cm^{-1}) as a function of wavelength, and $F(\lambda)$ is the corrected fluorescence spectrum of the donor probe. Limits on κ^2 can be established by taking into account both static and dynamic averaging among the donor-acceptor pairs (see Results). A typical distance D between donor and acceptor pairs in the micelle system, with a surface density of one acceptor probe per micelle is

$$D^6 = R_o^6(T^{-1} - 1)\,, \tag{4}$$

where T is the resonance transfer efficiency.

FRET distance in detergent model system

We have used a model (Fig. 2) to estimate a typical distance between a fluorescent donor and an acceptor anchored in the micelle. SBP (391.6 mol wt) forms micelles with a radius of ~20 Å and an aggregation number (n_{agg}) of 155 (Herrmann, 1966). The calculated micelle surface area of 5000 $Å^2$ results in a projected head group area of the detergent molecule of approximately 32 $Å^2$. At 100 μM SBP, 85 μM of the detergent was estimated to form micelles (see Fig. 3). If FITC molecules on the LPS polysaccharide chain are at a distance d from the micelle surface, the FRET distance D between donor and acceptor pair represents a typical distance where the micelle radius is r and

$$d^2 = D^2 - r^2\,. \tag{5}$$

The distribution of acceptor molecules inserted in the micelles was assumed to follow a Poisson distribution (Gennis and Cantor, 1972; Estep and Thompson, 1979):

$$P(k) = e^{-L}L^k/k!\,, \tag{6}$$

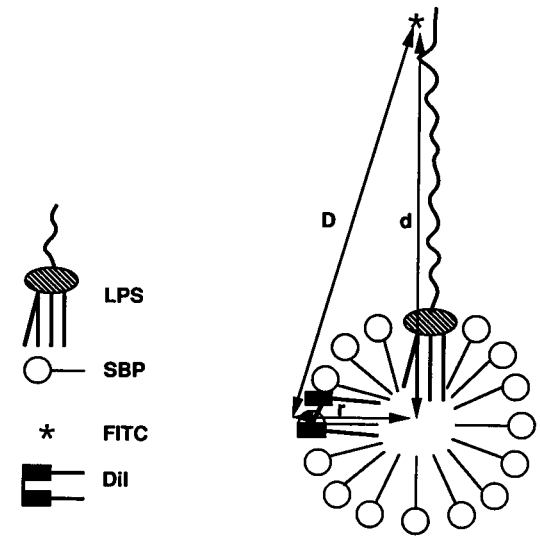

FIGURE 2 Micelle resonance energy transfer model. The SBP detergent micelle is shown with a radius of ~20 Å, FITC-labeled polysaccharide linked to a lipid A molecule (LPS) as donor, and a micelle-partitioned C18-DiI as acceptor (R_o = 68 Å).

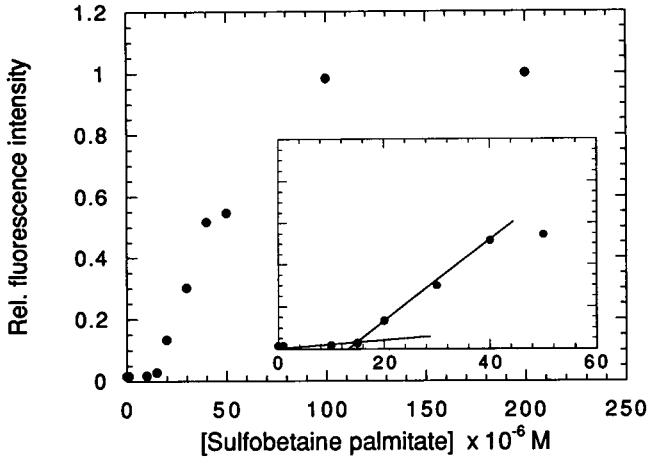

FIGURE 3 CMC determination for SBP with NPN, with fluorescence emission intensity at 425 nm of 5 μM NPN as a function of concentration for *N*-dodecyl-*N,N*-dimethyl-3-ammonio-1-propane sulfonate (SBP), at 20°C, in 0.02 M TRIS, 0.15 M NaCl at pH 7.5. Excitation was at 350 nm. The intensity of NPN increases sharply in the range of CMC. In the inset, the CMC can be found where the lines of the two slopes intercept.

where L is the average number of DiI molecules per detergent micelle, k is the number of DiI molecules per micelle, and $P(k)$ is the number of micelles with k DiI molecules inserted. When there is an average of one acceptor/micelle, $P(0) = 0.37$, $P(1) = 0.37$, and $P(2 \text{ or more}) = 0.26$. It can be shown that the decrease in steady-state fluorescence due to energy transfer follows the general form

$$F_a/F_0 = e^{-L} + (1 - e^{-L})(1 - E), \qquad (7)$$

where F_a/F_0 is the ratio of fluorescence in the presence of acceptor to fluorescence in the absence of receptor and E is the energy transfer efficiency (Jones, Wiström, Wofsy, and Sklar, in preparation).

RESULTS

Characterization of the labeling of FITC-LPS

We initially characterized the commercial LPS according to the following two criteria: association of the FITC with the LPS and fluorescence of the aggregate before and after exposure to detergent. Association of the LPS was verified by essentially quantitative precipitation of fluorescence with LPS aggregates (88–96%) and co-migration of fluorescence with LPS on carbohydrate-stained deoxycholate-PAGE (Tsai and Fraisch, 1982; Komuro and Galanos, 1988). There was no evidence of contaminating fluorescent protein (data not shown). Wild-type LPS (i.e., *S. minnesota* and *S. abortus equi*), which is heterogeneous, contained several fluorescent gel bands. When we substituted the deoxycholate with SBP in the PAGE buffer, the FITC-LPS did not separate on the gel.

Initial measurements of FITC-LPS from short-chain *S. minnesota* Re 595 indicated self-quenching within the FITC-LPS aggregates. By measuring the fluorescence of the FITC-LPS before and after solubilization in SBP detergent buffer, we found that fluorescence quenching was relieved from low molecular weight FITC-LPS aggregates (Table 1).

TABLE 1 Fluorescence from FITC-LPS aggregates relative to micelles

FITC-LPS strain	MW	% fluorescence ± SE
S. minnesota	20,000	99.1 ± 0.5
S. typhimurium	7,000–80,000*	86.0 ± 1.9
E. coli 0111:B4	10,000	85.7 ± 4.5
Se. marcescens	ND	69.1 ± 1.7
S. abortus equi	5,000–10,000‡	60.0 ± 0.9
S. minnesota Re 595	2,500	22.8 ± 0.8

Fluorescence intensity was measured at 520 nm before and after solubilization in 100 μM SBP, at 20°C, in 0.02 M TRIS, 0.15 M NaCl, pH 7.5. MW, molecular weight; SE, standard error of n = 3–6 experiments; ND, not determined.

*Molecular weight from Palva and Mäkelä, 1980. Molecular weights for mutant and wild-type LPS were not determined in this study.

‡Molecular weight from Komuro and Galanos, 1988. Molecular weights for mutant and wild-type LPS were not determined in this study.

The short polysaccharide chain mutant strain *S. minnesota* Re 595 (2500 mol wt) was highly self-quenched compared with FITC-LPS from other bacterial strains with ~23% fluorescence before solubilization. The FITC-LPS from *S. minnesota* (with a long polysaccharide chain, 20,000 mol wt) exhibited no self-quenching whereas the fluorescence for intermediate size FITC-LPS aggregates varied in intensity between 60 and 86%.

Characterization of detergent micelles and incorporated probes

The CMC of SBP was measured by the NPN method. A CMC of 15 μM was estimated from Fig. 3. This value is in agreement with Brito and Vaz (1986). SBP is a suitable detergent for these experiments as the micelles form at low concentrations. Because the total micelle concentration is ~0.6 μM, multiple acceptor probes can be introduced per micelle without optical artifact. We anticipated that FITC-LPS would be inserted and anchored to the detergent micelle by the lipid A unit whereas the FITC-labeled polysaccharide chain would extend into the surrounding medium. Fluorescence lifetime measurements of FITC-LPS in micelles are dominated by the native FITC lifetime (see Table 3). These lifetime results are consistent with monomeric FITC-LPS solubilized in SBP, where the FITC is exposed to the aqueous environment. At the SBP concentration used, optimal solubilization of the probes was suggested because no further fluorescence increases were detected when the SBP concentration was increased.

Stern-Volmer quenching of the probes in SBP was measured in the presence of KI (data not shown). The quenching of free fluorescein not incorporated into SBP and the long-chain wild-type *S. minnesota* FITC-LPS molecules in the micelle showed linear dynamic quenching. The quenching constant of the long-chain wild-type *S. minnesota* LPS species in SBP, where the probe is extended away from the micelle surface, was reduced by approximately one-half, as expected based on mobility (Eftink, 1991). In contrast, the

quenching of probes more closely associated with the micelle (C18-Rhd, C18-fluorescein, DiI, *S. abortus equi*-FITC, and Re595-FITC) was complex, and the initial rate of quenching was 2 to 10 times more efficient than expected. To determine whether there was specific association of the iodide with the micelles, we further examined the quenching of 2- and 12-(9-anthroyloxy)-stearic acids. These probes both showed more efficient quenching than expected from diffusion alone. Because probes suspected of being at the surface or in the micelle interior were quenched by iodide, the precise probe location could not be assigned.

Resonance energy transfer in SBP

Fig. 4 shows the absorption and emission spectra of FITC-LPS from *S. minnesota* Re 595 and the absorption spectrum of C18-DiI incorporated into detergent micelles. Table 2 summarizes J, κ^2 ranges, and R_0 of relevant donor-acceptor pairs discussed in this study. For $\kappa^2 = 2/3$, the R_0 for FITC-LPS from *S. minnesota* Re 595 and for the *S. minnesota* was calculated to be 51 and 52 Å, respectively. These distances are comparable to those reported for carboxyfluorescein (Chen and Knutson, 1988). The R_0 for FITC-LPS and C18-DiI and C18-Rhd are 68 and 58 Å, respectively. Limitations on the range of κ^2 can be established by considering both dynamic reorientation of the probes and static averaging of their orientation (Dale and Eisinger, 1974; Dale et al., 1979; Eftink, 1991; Wu and Brand, 1992). Polarization measurements were used to estimate the mobility of the probes, allowing upper and lower bounds of κ^2 to be set. These upper and lower bounds of κ^2 translate into uncertainties of approximately 15% in the values of R_0 (see Table 2).

Fig. 5 shows the FRET between C18-fluorescein or FITC-LPS donors and C18-DiI (Fig. 5 *A*) or C18-Rhd (Fig. 5 *B*) acceptors as a function of the average number of acceptors per micelle. Also shown are fits to this data using Eq. 7. The quenching of free fluorescein was used as a control to evaluate inner filter (competing absorption of exciting light) or outer filter (absorption of emitted light) effects associated with increased acceptor concentration. The energy transfer between C18-fluorescein and the acceptors serves as a control to calibrate the extent of FRET that occurs when donors and acceptors are constrained to the micelle, presumably near the surface. Qualitatively, the results show that 1) FRET of free FITC is minimal in this system; 2) the location of the mutant *S. minnesota* Re 595 FITC-LPS and C18-fluorescein are similar relative to the micelle surface; 3) the FITC conjugated to wild-type *S. minnesota* LPS is largely out of range of energy transfer with the acceptors on the micelle surface; 4) heterogeneous and intermediate length LPS (i.e., *S. abortus equi, E. coli* 0111, *Se. marcescens* and *S. typhimurium*) exhibit partial energy transfer, intermediate between the short-chain and the long-chain extremes.

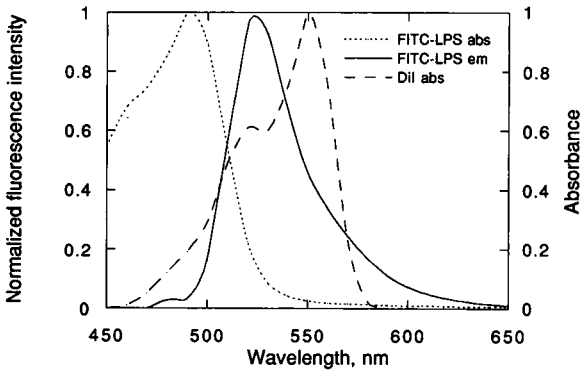

FIGURE 4 Spectral overlap of FITC-LPS and C18-DiI. The absorbance spectrum of 30 ng/ml FITC-LPS from *S. minnesota* Re 595 (· · ·), fluorescence emission intensity of 30 ng/ml FITC-LPS from *S. minnesota* Re 595 (——), and the normalized absorbance spectrum of C18-DiI at one molecule per micelle (– – –), all in 100 μM SBP, 0.02 M TRIS, 0.15 M NaCl buffer at pH 7.5 at 20°C, is shown. The FITC-LPS was excited at 470 nm and the fluorescence peak was normalized to 1.0.

Fluorescent lifetimes

Time-resolved fluorescence spectroscopy was used to examine the lifetime of the FITC label in LPS aggregates before and after solubilization into detergent micelles (Table 3 and Fig. 6). Several bacterial strains of FITC-LPS were chosen: the short-chain *S. minnesota* Re 595, the long-chain *S. minnesota* wild type, and two heterogeneous intermediate-chain wild-type species, *S. abortus equi* and *Se. marcescens*. We observed that only the short-chain, highly quenched FITC-LPS aggregates in the absence of detergent exhibit substantially reduced lifetimes compared with native FITC. The results are likely to represent extensive transfer interactions in the aggregates that diminish as the LPS chains increase in length. For all of the LPS species in detergent, the native FITC lifetime predominates. The source of the short lifetime components of solubilized FITC-LPS in detergent is not known.

After addition of up to at least two DiI molecules per micelle, the lifetimes of all LPS species exhibited the same characteristic behavior in which the native FITC lifetime always dominated. We would expect to observe intermediate fluorescent lifetimes if there were a large fraction of FITC at a distance near R_0 or if a large fraction of the FITC were varying in distance from the micelle surface over the FITC lifetime. Qualitatively, the observations suggest that energy transfer was essentially all or none, in that it appears that FITC was either quenched (and exhibited a short or 0-ns lifetime) or remained unquenched (and exhibited its native lifetime).

Semiquantitative distance analysis

The micelle energy transfer system is unique in several respects. First, because the diameter of the micelle is small compared with R_0 for the donor-acceptor pairs in this study, donors near the surface will transfer efficiently regardless of

TABLE 2 Spectral overlap (J), dipole-dipole orientation factor (κ^2), and corresponding Förster transfer distances (R_0) for donor and acceptor pairs

Donor	Acceptor	J ($\times 10^{-13}$) cm^3 M^{-1}	κ^2	R_0 (Å)
FITC-LPS *S. minnesota* Re 595	FITC-LPS *S. minnesota* Re 595	0.9	2/3*	51
FITC-LPS *S. minnesota* Re 595	C18-DiI	5.3	0.2–1.7	57–79
FITC-LPS *S. minnesota* Re 595	C18-rhodamine	2.3	0.2–1.6	48–68
FITC-LPS *S. minnesota*	FITC-LPS *S. minnesota*	1.1	2/3*	52
FITC-LPS *S. minnesota*	C18-DiI	5.0	0.3–1.4	59–76
FITC-LPS *S. minnesota*	C18-rhodamine	2.0	0.3–1.3	51–65
C18-fluorescein	C18-fluorescein	1.6	2/3*	56
C18-rhodamine	C18-rhodamine	1.9	2/3*	57
C18-DiI	C18-DiI	2.8	2/3*	61
C18-fluorescein	C18-rhodamine	2.9	0.2–1.7	50–71
C18-fluorescein	C18-DiI	5.6	0.2–1.7	56–81

Upper and lower limits for κ^2 were generated using experimentally derived donor and acceptor anisotropies in combination with Dale et al. (1979; Fig. 9). Anisotropy values for fluorescent conjugates in 100 μM SBP: FITC-LPS *S. minnesota* Re 595, 0.158 ± 0.008; C18-fluorescein, 0.170 ± 0.001; C18-DiI (Fast-DiI), 0.230 ± 0.022; FITC-LPS *S. minnesota* wild type, 0.083 ± 0.002; C18-rhodamine, 0.215 ± 0.003.
*Illustrative values for self-transfer are estimated for dynamic randomization of probes.

how they are distributed over the micelle whereas donors removed from the surface greater than ~1.5 R_0 will transfer inefficiently regardless of the acceptor location in or on the micelle. Second, statistical arguments make it clear that, at an average of one acceptor per micelle, there is a distribution of approximately 37% of micelles with no acceptor and 63% with one or more acceptors. The third issue relates to the heterogeneity in FITC location along the LPS structure. It is important to recognize that there are several potential FITC labeling sites in the LPS molecules that we have not defined biochemically. However, it can be clearly recognized that 1) a similarity of transfer between C18-fluorescein and *S. minnesota* Re 595 LPS requires a similar location, likely to be near the micelle surface; 2) the inability to obtain efficient transfer or significantly reduced lifetime with the wild-type *S. minnesota* requires typical distances of separation in excess of 1.5 R_0; and 3) the observation of intermediate quenching without detecting intermediate lifetimes suggests that the mid-length chains exhibit some labeling sites that are within range of the micelle surface and some sites that are out of range, consistent with the idea that the oligosaccharide chain extends considerable distances from the micelle surface.

DISCUSSION

Characterization of LPS

LPS extracted from wild-type gram-negative bacteria are heterogeneous and vary in molecular weight. This heterogeneity results from the number of repeating saccharide units in the O-antigen polysaccharide and substitutions in the fatty acid chains of the lipid A unit. It has been shown, for example, with deoxycholate and sodium dodecyl sulfate gel electrophoresis that LPS of the smooth wild-type form of *S. minnesota* is composed of several molecular weight bands, suggesting that many different sizes of LPS are present in a single preparation (Peterson and McGroarty, 1985). In contrast, there are low molecular weight mutants of *S. minnesota* Re 595 that have single molecular weight bands (Komuro and Galanos, 1988; Komuro and Nakazawa, 1993). FITC is known to conjugate to amine groups that are present in the LPS structure. Free amines are present in the lipid A structure of several *Salmonella* strains (Wollenweber et al., 1984). We are confident that the bulk of the FITC present in the LPS preparations is associated with FITC-LPS structures because we found no visible contamination in the FITC-LPS samples either from protein or other molecules that could have been labeled with FITC (gels not shown) by deoxycholate PAGE. We also quantified free FITC in all FITC-LPS strains used in this study by using calcium to precipitate LPS aggregates (Galanos and Luderitz, 1975) and found the quantities low enough not to significantly interfere with the analysis.

There is a characteristic self-quenching in LPS aggregates, relieved in SBP, which depends upon the length of the oligosaccharide chain and the FITC/LPS ratio. The extent of self-quenching tends to be greater in the shorter-chain LPS species and is likely to be related to the proximity of fluoresceins in the aggregate. Even though lifetime and intensity measurements are available, it is difficult to interpret the self-quenching precisely because 1) the quantitative relationship between the observed fluorescence characteristics and the geometrical relationship between multiply transferring chromophores is difficult to calculate even when the geometry is known; 2) the molecular organization within the aggregates in suspension is poorly understood; and 3) the sites of labeling and their heterogeneity are not known. Although no definitive explanation is available for the self-quenching, the data are consistent with the idea that in the shorter chains the interactions involve structures in which the fluoresceins are arrayed in two dimensions, whereas in the longer-chain species the fluorescein extends to a third dimension representing a separation between lipid A and oligosaccharide chains.

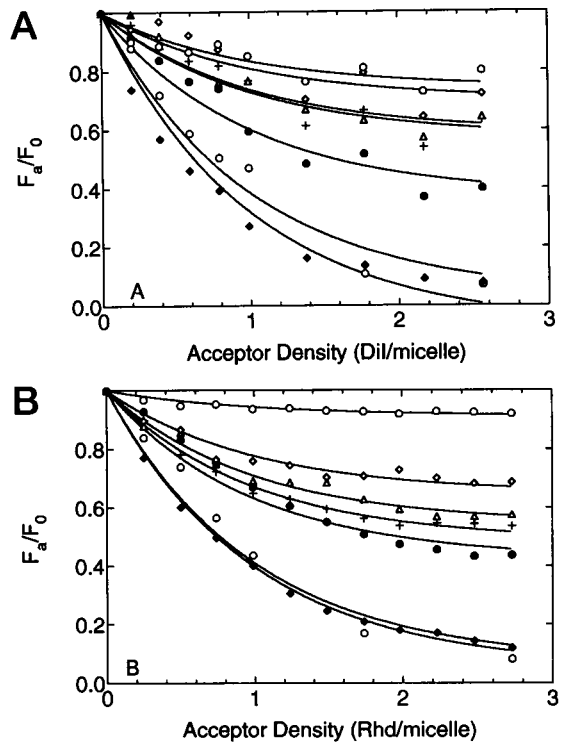

FIGURE 5 Quenching of C18-fluorescein and FITC-LPS in micelles by FRET to acceptors. (*A*) C18-DiI. (*B*) C18-Rhd. Data are shown as relative fluorescence (F/F_0) versus number of acceptors per micelle. Emission was at 520 nm from different strains of FITC-LPS (30 ng FITC-LPS/ml); excitation wavelength was 470 nm. The FRET data for the FITC-LPS and C18-fluorescein were corrected for quenching of free fluorescein as donor. ◆, *S. minnesota* Re 595; ○, *S. minnesota*; ◇, *S. typhimurium*; ●, *S. abortus equi*; +, *Se. marcescens*; △, *E. coli* 0111:B4; dotted circle, C18-fluorescein; in 100 μM SBP at 20°C with 0.02 M TRIS, 0.15 M NaCl buffer at pH 7.5. Energy transfer efficiencies for C18-DiI and C18-rhodamine, respectively, were found by fitting the data with Eq. 7 (*S. minnesota*, 0.26, 0.09; *S. typhimurium*, 0.30, 0.35; *E. coli* 0111:B4, 0.42, 0.46; *Se. marcescens*, 0.43, 0.52; *S. abortus equi*, 0.63, 0.58; C18-fluorescein, 0.97, 0.93; *S. minnesota* Re595, 1.07, 0.95).

The SBP micelle system

SBP was chosen because of its low CMC, which makes it possible to exhibit most of the detergent in micelles (effective concentrations of micelles is ~0.6 μM) at concentrations low enough that several acceptors can be incorporated per micelle without significant optical artifacts. At these SBP concentrations, the donors and acceptors appear to be randomly inserted in the micelles.

Previous authors have suggested that charged fluorophores associated with lipids should be exposed to the aqueous compartment (Sklar et al., 1980; Arias et al., 1993) and therefore expected to be at the micelle-water interface. We attempted to use quenching by KI as a qualitative verification of the exposure of the fluorophores. The Stern-Volmer analysis was complex and the fluorophores used in these studies were quenched to a variable extent. All of the probes expected to be associated with the micelle were quenched 2 to 10 times more efficiently than expected, leading to the possibility that iodide associated with or partitioned into the micelle. This idea was supported by the efficient quenching of the two anthroyloxystearic acid probes, one expected to be near the micelle surface and the other expected to be near its interior (Blatt and Sawyer, 1985). There was a suggestion of nonlinearity of the intermediate-chain LPS species. The increased efficiency of the initial quenching of LPS would be consistent with a fraction of the labeling at LPS sites associated with the micelle and iodide partitioning into the micelle.

Semiquantitative analysis of FRET in SBP micelles

The extent of the transfer depended upon the size of LPS to which the FITC was conjugated. The smallest conjugate of all, C18-fluorescein, and the *S. minnesota* Re 595 FITC-LPS gave efficient and comparable transfer. In contrast, transfer is limited with the longest chain, *S. minnesota*, and barely more efficient than that associated with acceptors in SBP and free fluorescein in solution. Intermediate quenching was observed with the LPS species with intermediate molecular weight chains. Fluorescence lifetime measurements were made to interpret the transfer in terms of distances. It was expected that FITC donors located at different distances from the micelle would have different lifetimes: distances less than 0.5 R_0 would result in very short lifetimes in the presence of acceptors; distances greater than ~1.5 R_0 would result in native (unquenched lifetimes); and intermediate distances would result in intermediate lifetimes. In all cases, the native lifetimes dominated and were accompanied by a short lifetime. Intermediate lifetimes were not detected. We interpret these data as evidence for donor-acceptor separations of less than 0.5 R_0 and greater than 1.5 R_0.

Because R_0 for the donor-acceptor pairs is considerably larger than the micelle diameter, an acceptor placed anywhere within the micelle should efficiently quench a donor in or on the micelle. In the case of C18-fluorescein, it means that the transfer efficiency is approximately a measure of the number of micelles that have incorporated an acceptor. Thus, at a concentration of one DiI/micelle, a quenching efficiency of 60% arises from the fact that ~40% of the micelles have no DiI whereas the remaining 60% have one or more. We expect the *S. minnesota* Re 595 FITC-LPS to be anchored in the micelle by its lipid A tail. Therefore, the similar efficiency of transfer as C18-fluorescein implies that the labeling site(s) are likely to be amines in the lipid A acyl chains or ethanolamines in the head group region. The quenching efficiency of donor fluorescence very nearly follows the Poisson distribution of acceptor in detergent micelles.

In contrast, the FRET from the wild-type *S. minnesota* indicates, to a very good approximation, that the labeled sites are predominantly out of range of the micelle surface. As the micelle diameter is small compared with the R_0, the

TABLE 3 Normalized steady-state and time-resolved fluorescence emission intensity of FITC-LPS aggregates and detergent micelles

	LPS aggregates				Detergent micelle*				1 DiI acceptor*				2 DiI acceptors*			
FITC-LPS	%SS	τ (ns)	FI	χ^2	%SS	τ (ns)	FI	χ^2	%SS	τ (ns)	FI	χ^2	%SS	τ (ns)	FI	χ^2
S. minnesota Re 595	27	3.29	0.69	1.1	100	3.96	0.83	0.1	39	3.82	0.77	0.5	27	3.77	0.75	0.7
		0.69	0.31			0.80	0.17			0.62	0.23			0.58	0.25	
S. minnesota	100	3.84	0.90	0.5	100	3.93	0.86	0.3	89	3.86	0.87	0.3	73	3.73	0.89	0.2
		1.09	0.10			1.29	0.14			1.12	0.13			0.91	0.11	
S. abortus equi	59	3.49	0.86	1.8	100	3.97	0.87	0.4	50	3.84	0.85	1.0	30	3.74	0.83	0.7
		0.60	0.14			0.98	0.13			0.65	0.15			0.49	0.17	
Se. marcescens	81	3.84	0.89	0.3	100	3.91	0.93	0.4	66	3.84	0.90	0.3	49	3.81	0.88	0.3
		0.99	0.11			0.77	0.17			0.70	0.10			0.76	0.12	

The fractional intensity (FI) of the lifetime (τ) was fitted to a two-component discrete analysis. SS, steady-state fluorescence emission at 520 nm; τ, lifetime. *100 μM SBP, at 20°C, in 0.02 M TRIS, 0.15 M NaCl, pH 7.5.

FRET occurs between donors extended out from the micelle and acceptor probes in the micelle, and most likely on the surface (Fig. 2). The FRET typical distance (D) between an acceptor and a donor FITC-LPS is comparable to d, the vertical distance between the FITC-LPS donor probe and micelle resident acceptor probe when one acceptor is present. Given the idea that there is effectively no reduction in the fluorescence lifetime of the donor, some of the FITC donors must be entirely out of range, or a minimum of 1.5 R_0 (8% transfer or lifetime reduction). These distances could extend well beyond 100 Å. The intermediate quenching of the intermediate length chains could arise from heterogeneity in the chains as well as the labeling sites. However, the predominance of the native lifetime argues again for the idea that labeling sites may extend a considerable distance from the micelle.

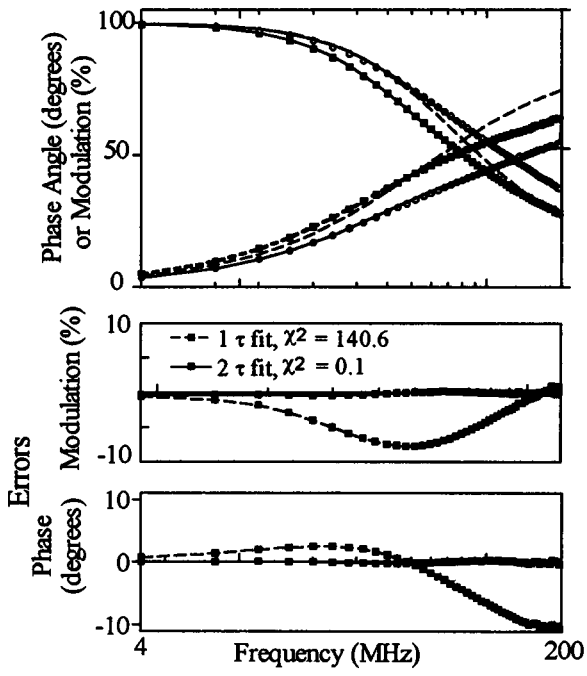

FIGURE 6 Fluorescence lifetime analysis. (*Top*) Frequency response of donor FITC-LPS from *S. minnesota* Re 595 native aggregates (○) and after solubilization in 100 μM SBP (■) at 20°C, in 0.02 M TRIS, 0.15 NaCl buffer at pH 7. The data are fitted to a two-component lifetime model. A single-lifetime component fit of FITC-LPS *S. minnesota* Re 595 in detergent is shown (- - -). (*Middle*) Residual error of lifetime modulation for one-component fit (– – –) and the two-component lifetime fit (——) to the frequency response of native FITC-LPS *S. minnesota* Re 595 after solubilization in SBP. (*Bottom*) Residual error of frequency response to phase angle in degrees in FITC-LPS *S. minnesota* Re 595 upon solubilization in SBP.

We have calculated the distribution of lifetimes that are expected if the donor is at a fixed distance and the acceptors are randomly distributed over the surface of individual micelles (Jones, Wiström, Wofsy, and Sklar, in preparation). These calculations suggest that intermediate lifetimes will be detected when a considerable fraction of the donors are clustered at a distance comparable to R_0 from the surface. If the donors are distributed heterogeneously, then the closer ones are nearly fully quenched whereas the farther ones are barely quenched. In this case, there would be steady-state quenching of fluorescence intensity while the native lifetime would continue to dominate. It is also possible to obtain a distributed intermediate lifetime under conditions where there is motion of donors relative to acceptors on the time scale of the donor emission (Lakowicz et al., 1987). This could occur by diffusion in the micelle or by flexibility of the oligosaccharide chain. No evidence for either of these has yet been established.

The results presented here suggest that, when FITC-LPS is solubilized into SBP micelles, information can be obtained about the extended conformation of the FITC-LPS with longer O-specific chains. We hope to use such systems, which are spectroscopically well behaved compared with LPS aggregates, to investigate the interaction of LPS with its binding molecules for the initiation of the septic shock pathway. The use of FRET in micelles is also a potential tool for the evaluation of macromolecular assemblies involving membrane and soluble components.

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