Analysis of the binding of polymyxin B to endotoxic lipid A and core glycolipid using a fluorescent displacement probe

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Dansylcadaverine, a cationic fluorescent probe binds to bacterial lipopolysaccharide and lipid A, and is displaced competitively by other compounds which possess affinity toward endotoxins. The binding parameters of dansylcadaverine for lipid A were determined by Scatchard analysis to be two apparently equivalent sites with apparent dissociation constants \(K_d\) ranging between 16 \(\mu M\) to 26 \(\mu M\), while that obtained for core glycolipid from Salmonella minnesota Re595 yielded a \(K_d\) of 22 \(\mu M\) to 28 \(\mu M\) with three binding sites. The \(K_d\) of polymyxin B for lipid A was computed from dansylcadaverine displacement by the method of Horovitz and Levitzki (Horovitz, A., and Levitzki, A. (1987) Proc. Natl. Acad. Sci. USA 84, 6654–6658). The applicability of this method for analyzing fluorescence data was validated by comparing the \(K_d\) of melittin for lipid A obtained by direct Scatchard analysis, and by the Horovitz-Levitzki method. The displacement of dansylcadaverine from lipid A by polymyxin B was distinctly biphasic with \(K_d\) for polymyxin B–lipid A interactions corresponding to 0.4 \(\mu M\) and 1.5 \(\mu M\), probably resulting as a consequence of lipid A being a mixture of mono- and di-phosphoryl species. This was not observed with core glycolipid, for which the \(K_d\) for polymyxin was estimated to range from 1.1 \(\mu M\) to 5.8 \(\mu M\). The use of dansylcadaverine as a displacement probe offers a novel and convenient method of quantitating the interactions of a wide variety of substances with lipid A.

Introduction

Lipopolysaccharides (LPS) are major components of the outer membrane of gram negative bacteria and elicit endotoxic and immunologic activities in susceptible hosts [1–3]. The lipid A moiety of LPS constitutes the active principle of endotoxin [4–7]. Melittin, an amphiphilic, basic polypeptide from bee venom binds to lipid A and attenuates its endotoxicity [8]. The binding of melittin to lipid A was characterized using the intrinsic fluorescence of the lone tryptophan residue of the polypeptide. It was necessary to develop a suitable method to characterize the interactions with lipid A of a variety of compounds which are not intrinsically fluorescent, in order to identify molecules with potential therapeutic value. In this report, we describe the use of a fluorescent probe, dansylcadaverine, which binds to lipid A and is displaced by other substances which exhibit affinity for lipid A. The differences in the spectral properties of bound and displaced (free) dansylcadaverine affords a novel and convenient means of characterizing the binding of compounds to lipid A. The method was validated using melittin as a displacer. The binding of polymyxin B to lipid A, and to core glycolipid from the deep rough mutant of Salmonella minnesota was analyzed using this method.

Materials and Methods

Polymyxin B sulfate, melittin from Apis mellifera, lipid A and core glycolipid from Salmonella minnesota strain Re 595 (CGL) were purchased from Sigma Chemicals (St. Louis, MO). Melittin was further purified as described earlier [8]. The concentration of melittin in solution was calculated by assuming a molar extinction coefficient value of 5570 \(M^{-1}\) at 280 nm [9]. Re core-glycolipid is lipopolysaccharide extracted from deep-rough mutant bacteria and is composed of lipid A and two to three molecules of an anionic eight carbon sugar residue, 3-keto-2-deoxyoctonate (KDO) [10].
Aqueous stock suspensions of lipid A (2.5 mg/ml) and CGL (5 mg/ml) were prepared by sonication with trace quantities of triethyamine.

Dansylcadaverine (n-(5-aminopentyl)-5-dimethylamino-1-naphthalene sulfonamide, DC) was synthesized as described elsewhere [11]. The compound was verified to be homogeneous by TLC on silica-gel using petroleum ether/chloroform/methanol (2:2:1). 270 MHz 1H NMR, IR and UV spectra confirmed the structure. Concentrations of DC in methanolic solutions were determined by spectrophotometry at 330 nm using a molar extinction coefficient of 3.300 M⁻¹ for the dansyl moiety.

Fluorescence experiments were performed on a Shimadzu RF 5000 spectrofluorimeter at 25°C. The excitation wavelength was 340 nm. Emission spectra were recorded with bandpass values of 5 nm for both monochromators. Steady-state emission polarization parameters were acquired at band pass values of 10 nm and anisotropy and polarization were calculated according to Shinitzky and Barenholz [12]. Fluorimetric titrations were carried out in 100 mM phosphate-buffered saline (pH 7.4) by addition of concentrated aliquots of probe, lipid A or CGL to avoid dilution effects, such that in the resulting mixture, the concentration of only one component was varied.

Scatchard analyses of the binding of the fluorescent probe to lipid A and CGL were performed by the methods of Azzi [13] and Madeira [14]. The determination of bound and free probe concentrations necessary for the construction of Scatchard plots by the method of Azzi requires a precise estimation of \( F_m \), the fluorescence yield of bound probe at unit probe concentration. This parameter is usually obtained by linear extrapolation of fluorescence intensity to infinite ligand concentration by means of a double reciprocal plot [13]. In these experiments, however, linear extrapolation was not possible as the double-reciprocal plots were distinctly nonlinear, and simple linear regressions led to underestimated \( F_m \) values from which Scatchard plots could not be constructed. This is not surprising in view of the complex aggregation states of lipid A in aqueous suspensions that probably behave as polyanionic surface lattices [15]; the nonlinearity would also mean that binding parameters obtained from this data would only represent empirical affinity constants and binding stoichiometries. In order to extract these empirical parameters from Scatchard analyses, an alternate procedure was used to estimate \( F_m \); a quadratic function was modelled on the data points using a Simplex function minimizing algorithm [16]. \( F_m \) was then obtained from the reciprocal of the Y-intercept of the curve divided by the probe concentration used. The values of parameters thus obtained were verified by an alternate method [14]. The latter method (referred to hereafter as Scatchard-type plots) yields only \( K_d \) and not the binding stoichiometry. The \( K_d \) values obtained by these two methods are consonant and thus affirms the validity of the approach used. Displacement experiments using polymyxin B were performed with a 10–12 molar excess of probe to ensure a high bound/free ratio of lipid A or CGL. The concentrations of probe used (typically 70–80 μM) did not result in any observable inner-filter effects. Various methods of analyzing displacement data were critically evaluated. The Cheng-Prusoff method [17] of analyzing displacement data was found to be inappropriate for these experiments since it requires that the concentrations of the probe and the displacer be in excess over receptor (lipid A) concentrations, which could not be fulfilled. The Horovitz-Levitzki method [18], originally applied to radioligand displacement studies, was therefore used for analyzing fluorescence displacement data, and was verified by comparing the dissociation constant values of melittin for lipid A obtained by Scatchard analysis [8] with that acquired by the Horovitz-Levitzki method. A modification was necessary to estimate occupancy, the fraction of lipid A (receptor) bound to DC (ligand) at various displacer (polymyxin B) concentrations; this parameter can be directly measured in radioligand experiments. Occupancy was therefore calculated as \( (F - F_o)/(F_{max} - F_o) \), where \( F_o \) is the fluorescence intensity of DC alone, \( F_{max} \) is the intensity in the presence of lipid-A, and \( F \) are the intensities of the DC-lipid A mixture at different displacer concentrations. The estimation of occupancy in this manner implies that this parameter assumes a value of 1 at zero displacer concentration (since \( F = F_{max} \)) and 0 when DC is completely displaced with excess of the displacing ligand \( (F = F_o) \). Thus, for fluorescence experiments, the values of this parameter are calculated, rather than explicitly measured as in radioligand displacement experiments [18].

Results

The binding of DC to lipid A and CGL

The addition of lipid A to DC results in a marked enhancement of fluorescence intensity accompanied by a blue shift in the wavelength of maximum emission (Fig. 1), which are indicative of interactions of DC with hydrophobic sites on the lipid A molecule. Triethylamine alone, had no effect on DC fluorescence even at concentrations several times higher than that in the lipid A suspension. Steady-state emission polarization values of DC also increase concurrently from 0.031 to 0.157, signifying complex formation.

The empirical binding parameters obtained for lipid A are represented in Fig. 2. The apparent stoichiometry of binding appears to be two equivalent binding sites for DC with an apparent \( K_d \) of 16.6 · 10⁻⁶ M; a
**Fig. 1.** Enhancement and blue-shift of DC fluorescence by lipid A. Dashed line: Emission spectrum of DC alone (10 μM) in PBS (pH 7.4). Solid lines: spectra of DC in the presence of indicated concentrations of lipid A. Inset: Double-reciprocal plot of fluorescence intensity as a function of lipid A concentration. Symbols indicate actual experimental data. The curve was fit by a quadratic model (see Methods section).

$K_d$ of $26 \times 10^{-6}$ M is obtained by the Scatchard type plot (Fig. 2, inset).

The addition of CGL to DC also results in fluorescence intensity enhancement and blue shift of emission maxima as with lipid A (data not shown). Scatchard analyses (also requiring nonlinear extrapolation of $F_m$) indicate three apparently equivalent binding sites for DC with apparent dissociation constants of $22 \times 10^{-6}$ M (Fig. 3) to $28 \times 10^{-6}$ M (Fig. 3, inset).

**Displacement experiments**

Fig. 4 (inset) shows the displacement of DC from lipid A as a function of melittin concentration. The slope of the Horovitz-Levitzki plot (Fig. 4) represents the $K_d$ of the displacing ligand (melittin), while the intercept on the ordinate axis, in the case of purely competitive, noncooperative binding is the $K_d$ of the displaced ligand (DC) for lipid A. The $K_d$ of melittin for lipid A, computed from the Horovitz-Levitzki plot (Fig. 4) is $3.9 \times 10^{-6}$ M, which is in good agreement with a $K_d$ of $2.5 \times 10^{-6}$ M obtained directly in our earlier study [8]. The $K_d$ of DC for lipid A indicated by the $Y$ axis intercept is $19.8 \times 10^{-6}$ M which is in good agreement with the values obtained by Scatchard analysis (Fig. 2).

The addition of polymyxin B to the mixture of lipid A and DC results in attenuation of fluorescence intensity and a red shift of emission maxima, while the addition of polymyxin B to DC alone elicits no such effects. This is indicative of the displacement of bound DC from lipid A by polymyxin. The displacement-induced quenching is represented in Fig. 5 (inset) as occupancy of lipid A by DC. The shape of the displacement curve is suggestive of two different classes of binding sites on lipid A for polymyxin, or alternatively, two species of lipid A molecules with differing affinities for polymyxin. Since the two phases of the curve...
are well separated, they were analyzed as displacements of two separate single-site systems [19]. The data represented in Fig. 5 yields apparent dissociation constants of $0.4 \cdot 10^{-6}$ M and $1.5 \cdot 10^{-6}$ M for the high–low affinity interactions respectively, of polymyxin B with lipid A. The ordinate axis intercept, representing the $K_d$ of DC for lipid A is $19.2 \cdot 10^{-6}$ M, which is in good agreement with that obtained by Scatchard analysis (Fig. 2), indicating that the binding of polymyxin to lipid A entails competition for sites occupied by DC.

The displacement of DC from CGL by polymyxin B is represented in Fig. 6 (inset). In contrast to lipid A, the displacement curve seems to correspond to that of one binding site. However, the Horovitz-Levitzki plot is non-linear (Fig. 6), with the slopes corresponding to apparent dissociation constants of $1.1 \cdot 10^{-6}$ M and $5.8 \cdot 10^{-6}$ M if the data points are resolved into two linear components. The ordinate axis intercept is $20.2 \cdot 10^{-6}$ M, consonant with values obtained from the Scatchard plots (Fig. 3).

Discussion

In this paper, we have presented evidence for the binding of DC to lipid A and CGL, characterized the binding parameters and outlined its use as a fluorescent probe to quantitate interactions of other compounds with lipid A or LPS, using polymyxin as an illustrative example. Polymyxin has been shown to form stable molecular complexes with the lipid A portion of LPS [20], and the binding which is thought to involve both electrostatic and hydrophobic interactions, results in almost complete abrogation of endotoxic activity [21]. We evaluated DC because of its basic and hydrophobic character which should facilitate its interaction with the acidic phosphate groups and apolar acyl components of lipid A. Indeed, the interaction of DC
with neutral phospholipids, dimyristoyl- and dipalmitoylphosphatidylcholine is much weaker (data not shown), suggesting that electrostatic interactions may play an important role in the binding of DC to lipid A. The charge-dependent differential binding would also be suggestive of true binding and not merely partitioning or adsorption of the probe.

The poor aqueous solubility, intrinsic microheterogeneity, complex phase behaviour and incompletely-characterized aggregation states of lipid A complicate, in no small measure, the study of the binding of the probe to lipid A. It must be emphasised that since the probe binds not to isolated monomers, but multimolecular lipid assemblies, unequivocal interpretation of binding parameters would not be possible, however sophisticated and rigorous techniques may be used to extract values of sites or affinity constants. However, empirical values provided by this method may be readily used to compare the relative binding affinities to lipid A of compounds, and may thus be used to screen substances for lipid A-binding behaviour.

It is interesting that the stoichiometry of two molecules of DC to one of lipid A obtained by Scatchard analysis would correspond to a conceptually feasible model of the DC-lipid A complex which envisages electrostatic interactions between the terminal phosphate residues on the disaccharide backbone of lipid A and the free amino group of DC, and also, hydrophobic interactions between the alkyl moiety of DC with the nonpolar acyl substituents of lipid A. It is not clear however, why CGL with its three additional anionic KDO groups provides only one more site for the binding of DC. A variety of cations are known to bind to LPS with dissociation constants in the range of 6 μM–15 μM [22]. Cation-binding to CGL and lipid A is also manifested by an increasing tendency to form nonlamellar, inverted cubic structures [15]. It is likely, therefore, that cation occupancy may modify the binding of DC to CGL. This may also be responsible for the non saturability of DC fluorescence even at very high lipid A/CGL concentrations which consequently required nonlinear curve fitting of double-reciprocal plots for determining $F_m$. Inaccuracies in $F_m$ estimations may account for the minimal discrepancies in $K_d$ obtained by the two methods. Deionized lipid A or LPS obtained by electrodialysis and converted to soluble uniform salt forms with triethylamine or tetraethylammonium hydroxide [23] may help resolve the problem of cation contamination, but such preparations were unavailable.

The displacement of bound DC by polymyxin afforded a convenient means of estimating empirical binding parameters. In the case of lipid A, the biphasic displacement curve may be a consequence of the sample being a mixture of mono- and diphosphoryl species. The acid-labile character of the glucosamine-1-phosphate linkage may cause considerable losses of phosphate during mild acid hydrolysis employed to liberate lipid A from LPS [24]. Phosphate analyses and biological potency in a previous study [8] are corroborative in this regard. The upward curvature of the Scatchard-type plot (Fig. 2, inset) may also be indicative of heterogeneity [25,26]. This may be verified using homogeneous, synthetic, mono- and diphosphoryl lipid A. The nonlinearity of the Horovitz-Levitzki plot for CGL (Fig. 5) is also likely to be due to sample heterogeneity and/or cation occupancy.

An earlier study [22] employing dansylated LPS derivatives reported a $K_d$ of 0.3 μM for polymyxin using a Re type rough strain of S. typhimurium; the lack of derivatizable groups on lipid A precluded estimations of $K_d$ for lipid A in this study. Dansylpolymyxin has been reported to bind both LPS and lipid A with identical affinities (Hill parameter, $S_0 = 0.38$ μM) [27]. An electron spin resonance probe [28] as well as dansylpolymyxin [29] have been used as displacement probes for analyzing cation interactions with LPS/lipid A, but binding parameters were not made available.

In conclusion, the use of dansylcadaverine as a displacement probe provides a convenient and simple method for analyzing interactions of various compounds with endotoxic lipid A. This technique may be employed to screen compounds for lipid A antagonistic properties preceding comprehensive evaluation in biological assays. Several basic amphiphilic peptides identified by this method to bind lipid A have also been shown to antagonise endotoxicity in biological assays (manuscripts in preparation).

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References


