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Characterization of complex formation between lipopolysaccharide and lysozyme

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ABSTRACT

The binding of lysozyme (LZM) to bacterial lipopolysaccharide (LPS) inhibited the biological activities of LPS as well as the enzymic activity of LZM. The mode of binding has been characterized by using dansylated LZM and enzyme inhibition. The binding of LPS to LZM significantly increased the fluorescence intensity (Fl-intensity) of the danyl group and was found to be time-dependent; the complex was produced gradually and became stabilized within 20 min at 37°, 10 min at 50°, and 1 min at 70°. The maximum level of binding was also dependent on the reaction temperature, and more complex was formed at higher temperatures. Complexation was strongly dependent on the salt concentration and was not observed at >0.5M NaCl. From collected evidence of the Fl-intensities of various dansyl derivatives and amphiphiles, it is concluded that LZM interacts with LPS by multiple binding-modes, the first being strongly related to the enzyme inhibition, the second being close to the Fl-intensity, and the third being dependent on the inhibition of immunopharmacological activities. For the amphiphiles used in this study, sodium dodecyl sulfate (SDS), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-propanesulfonate (CHAPSO), decansulfonic acid, and cardiolipin have binding modes similar to that of LPS.

INTRODUCTION

Endotoxic lipopolysaccharides (LPS) are well recognized as important contributing factors to the pathogenesis of Gram-negative infections[1,2]. These amphipathic macromolecules exist as a major constituent of the bacterial outer membrane and, following their release from the bacterium, have the capacity to interact with variety of host target-cells and molecules[3,4]. Furthermore, LPS is known to interact with a variety of soluble serum proteins, including high-density lipoproteins, serum-complement proteins, and albumin, and evidence has also been presented that LPS binds to a variety of cationic proteins and peptides such as histones, the antibiotic polymyxin B, myelin basic protein, tachyplesin, anti-LPS factor (ALF), the bactericidal permeability-increasing protein (BPI), LPS-binding protein (LBP), cationic antimicrobial protein (CAP), and lysozyme (LZM)[5–15]. Although the biochemical factors that define the capacity of LPS to interact with the host cells and proteins have not been fully elucidated, available evidence suggests that binding of lysozyme to LPS alters the

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enzymic activity of LZM as well as the biological activity of the LPS[12-15]. Difficulties in investigating the interactions of LPS with the host-cell components arise from the structural heterogeneity of LPS, such as smooth and rough variants. The smooth forms of LPS usually constitute mixtures having O-polysaccharide of various chain-lengths. The biologically active lipid A part also has some heterogeneity in its substituents. During studies on the interaction of LPS and LZM, we have observed the enhancement of fluorescence intensity of dansylated-LZM in the presence of LPS.

We show here the characteristics of interaction between LPS and LZM by using various dansylated derivatives under a variety of reaction conditions.

EXPERIMENTAL

Materials. — Egg-white lysozyme (LZM), lipopolysaccharide from *Escherichia coli* 0111 and *Salmonella minnesota* Re595, dansyl chloride, polymyxin B sulfate, and *Micrococcus lysodeikticus* were purchased from Sigma Chemical Co. Detergents used were: sodium 1-decanesulfonate (**1**); sodium octanoate (**2**); sodium dodecyl sulfate (SDS, **3**); sodium deoxycholate (**4**); lithium dodecyl sulfate (**5**); cetylpyridinium chloride (**6**); Cetavlon (**7**); dodecyltrimethylammonium bromide (**8**); Triton X-100 (TritonX, **9**); Nonidet P-40 (NP-40, **10**); Tween 20 (Tween, **11**); octyl β-D-glucopyranoside (**12**); 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, **13**); 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonate (CHAPSO, **14**); and cardiolipin (**15**).

Preparation of dansylated lysozyme (DNS-LZM). — LZM was labeled with 1-dimethylaminonaphthalene-5-sulfonyl(dansyl) chloride by published procedures[13] with slight modifications as follows: To a solution of LZM (35 mg) dissolved in 0.1M $NaHCO_3$ (25 mL), dansyl chloride (2 mg) in acetone (2.5 mL) was added at 4 °. The mixture was stirred gently overnight at 4°. The resulting nondialyzable fraction was passed through a column of Dowex 1-X8 resin (10 mL) equilibrated with 0.1M $NaHCO_3$, and the eluate was dialyzed against phosphate-buffered saline (PBS), pH 7.2, for 48 h. The resulting solution was frozen until use. The concentration of DNS-LZM was measured by the Lowry-Folin method.

Preparation of dansylated polymyxin B (DNS-PB). — Polymyxin B (PB) was labeled with dansyl chloride by published procedures[13] with slight modification as follows: To a solution of PB (100 mg) dissolved in 0.1M $NaHCO_3$ (10 mL), dansyl chloride (25 mg) in acetone (5 mL) was added at room temperature. The mixture was kept overnight at room temperature after sonication. The resulting solution was passed through a column of Dowex 1-X8 (10 mL) equilibrated with 0.1M $NaHCO_3$. The eluate and 60% ethanol eluate were pooled, and the combined solution was evaporated and dissolved in PBS.

Measurements of fluorescence spectra. — Fluorescence of DNS-derivatives were measured at constant temperature with a Hitachi 650-40 fluorescence spectrophotometer at the excitation wavelength of 340 nm, and emission spectra were monitored

from 440 to 560 nm. For quantitative or kinetic measurements, an emission wavelength of 500 nm was used.

Calculation of affinity constants. — Binding assays were performed in 1 mL of PBS, pH 7.2, and the amount of DNS-LZM bound to LPS was determined by using the following equation: amount of DNS-LZM bound = $(f/F_{max}) \times$ concentration of DNS-LZM. The f value was the observed fluorescence at the indicated DNS-LZM concentration using a subsaturating amount of LPS, and F_{max} was the fluorescence of DNS-LZM using a saturating amount of LPS (50 μg/cuvette). The amount of free DNS-LZM was determined by subtracting the amount of bound DNS-LZM from the total amount of DNS-LZM. The dissociation constant (K) was calculated from the following equation:

$$1/B = 1/n[LPS] \times K/F + 1/n[LPS]$$

were B, F, n, and [LPS] were the concentration of bound and free DNS-LZM, the number of binding sites, and the concentration of LPS, respectively.

Measurement of lysozyme enzymic activity. — The enzymic activity of lysozyme was assessed by the rates of lysis of *M. lysodeikticus*. Lysozyme solution (25 μL) dissolved in PBS (10 μg/mL) were mixed in a micro plate with or without LPS (1 mg/mL) (10 μL), with or without the addition of other materials. The mixture were incubated for 10 min at 37°. *M. lysodeikticus* cells (1 mg/mL) were added in a volume of 200 μL to the mixture and the initial velocities were determined by measuring the decreasing turbidity of *M. lysodeikticus* cells with a micro-plate reader (MTP-32, Corona Electric Co., Ltd., Tokyo) monitored at 630 nm.

RESULTS

Comparison of the binding of LZM and DNS-LZM to LPS. — In this paper, we characterize the binding of LZM to LPS by using the dansyl group as a fluorescent probe. The first experiment was designed to compare some properties of LZM to those of DNS-LZM. As described previously[13], the enzymic activity of DNS-LZM used in that paper was estimated to be 82% compared to native LZM, and the enzymic activities of both LZM and DNS-LZM were inhibited almost equally by the addition of LPS. Fig. 1 shows the enzymic activity of LZM and DNS-LZM preparations used in this paper in the presence and absence of LPS. The enzymic activity of LZM and the sensitivity to LPS were not significantly changed by dansylation. (Repeated experiments showed that differences of the enzymic activity and the inhibition of activity between LZM and DNS-LZM were statistically not significant.) Additionally, as described later in Table I, Fig. 9 and Fig. 10, the Fl-intensity of DNS-amino acids was not be increased by LPS but was increased by some detergents, suggesting that the dansyl group itself does not significantly contribute to or alter the complex formation between LZM and LPS. However, it is noteworthy that refrigerating both of the dilute enzyme solutions gradually inactivated the enzyme, and thus the activity showed relatively large standard

deviations in each experiments. Decomposition of the DNS-substituent by light, a well-established phenomenon, would also cause a larger standard deviation.

In the previous study, we found that the fluorescence intensity (Fl-intensity) of DNS-LZM was significantly increased[13] in the presence of both smooth and rough LPSs. Fig. 2 shows representative fluorescence spectra of DNS-LZM in the presence and absence of LPS, and Fig. 2 (*inset*) also shows a representative dose-response curve of DNS-LZM in the presence of LPS. As discussed later more precisely, the Fl-intensity of DNS-LZM was significantly increased by the addition of LPS. To determine the specificity of the Fl-intensity, the parent LZM was mixed with DNS-LZM and the Fl-intensity was compared with that in the presence of LPS. As shown in Fig. 3, the Fl-intensity showed a dose-dependent decrease, suggesting the equivalence of binding between LZM and DNS-LZM. Considering these facts, monitoring the Fl-intensity of DNS-LZM may be useful for characterizing the binding of LZM and LPS. As discussed later, the ratio showing the highest Fl-intensity was ~1:2 (w/w, LZM/LPS). In contrast, inhibition of the enzymic activity required a greater amount of LPS, namely 1:20 (w/w, LZM/LPS). It is assumed that the complex is produced by relatively complicated processes.

Temperature dependence of the binding of LPS and LZM as assessed by the fluorescence intensity of DNS-LZM. — To examine the influence of reaction conditions on the interaction of LPS and LZM, the kinetics of the Fl-intensity were monitored at various temperatures. A final concentrations of 5 μg/mL of *E. coli* 0111 LPS and 5 μg/mL of DNS-LZM were used in this study. As shown in Fig. 4, the Fl-intensity depended significantly on the temperature. The Fl-intensity reached a maximum after 20 min or more incubation below 37°, 10 min at 50°, and 1 min at 70°. The maximum Fl-intensity was also dependent on the reaction temperature. Higher reaction temperatures gave higher Fl-intensities, except that, at 70° the Fl-intensity was lower than at 37° in the case of 0111 LPS, because (*a*) DNS-LZM itself exhibited a significantly elevated level of Fl-intensity at the higher temperature (time 0 in Fig 4a and b) and (*b*)

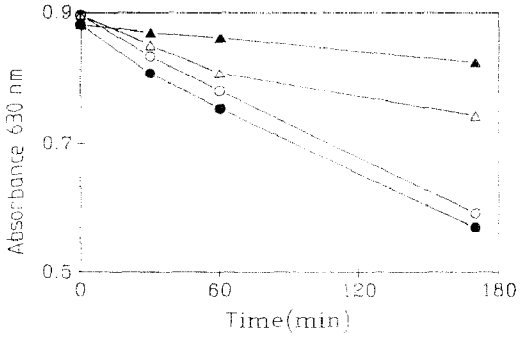

Fig. 1. Comparison of the enzymic activity of LZM and DNS-LZM in the presence or absence of LPS. [LZM or DNS-LZM (0.25 μg) were mixed with *E. coli* 0111 LPS (10 μg) and incubated for 10 min at 37°. Then, *M. lyso.* cells (200 μg) were added and turbidity was measured at appropriate intervals. ○, LZM; ●, DNS-LZM; △, LZM + LPS; ▲, DNS-LZM + LPS].

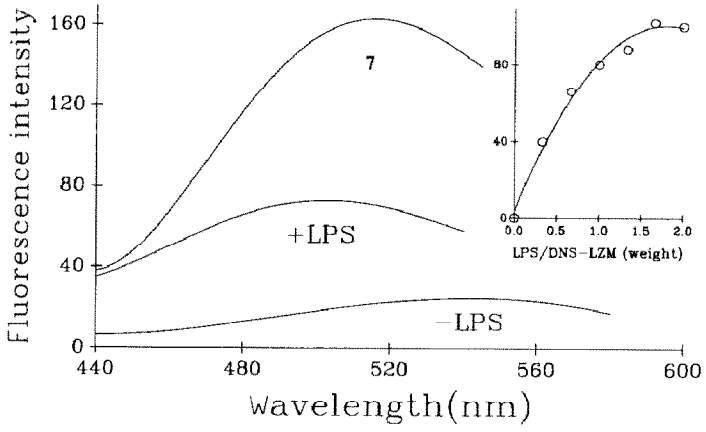

Fig. 2. Representative fluorescence spectra of DNS-LZM in the presence or absence of LPS or Cetavlon. [To a solution of DNS-LZM (5 μg in 1 mL of PBS), distilled water, *E. coli* 0111 LPS (5 μg) or Cetavlon (100 μg) were added. After 15 min of incubation at 37°, Fl-intensity was measured. The *inset* shows the dose–response curve of Fl-intensity of the LPS-DNS-LZM complex. To a solution of DNS-LZM (5 μg) in 1 mL of PBS, aliquots of *E. coli* 0111 LPS were added. After 15 min of incubation, Fl-intensity was measured as described in the section].

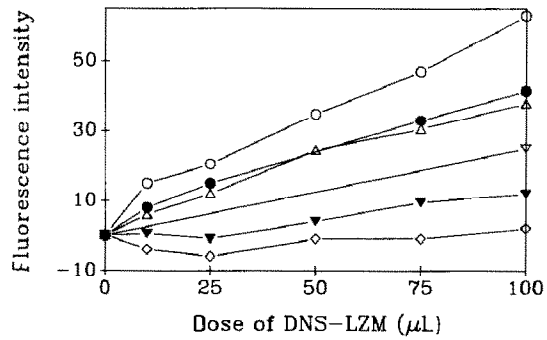

Fig. 3. LZM and DNS-LZM similarly bind to *E. coli* 0111 LPS. [Various quantities of LZM were mixed with DNS-LZM (5 μg) in 1 mL of PBS. To the mixture, 5 μg of LPS was added and it was incubated for 10 min at 37°. The Fl-intensities of the mixtures were measured as described in the section. DNS-LZM: LZM in ratio of 1:0 (○); 1:0.1 (●); 1:0.2 (△); 1:1 (▽); 1:2 (▼); 1:4 (◇)].

the conformation of LZM undergoes change around 70°. To clarify the temperature dependence of the Fl-intensity more precisely, after the complex conformation reached completion at each temperature, the temperatures of all reaction mixtures were changed to 25°, to compared the Fl-intensities at the same temperature. As shown in Fig. 5, the maximum of the Fl-intensity was also dependent not on the measurement temperature but on the reaction temperature, suggesting a higher, stronger, and irreversible binding at the higher temperature. These results suggest that binding of LPS and LZM involves not just one binding-site, but multiple binding-sites having different affinities. Thus, the binding is time- as well as temperature-dependent.

The fluorescence intensity of DNS-LZM was also significantly increased in the presence of rough (r-) LPS. Final concentrations of 5 μg/mL of *Salmonella minnesota* Re595 LPS and 5 μg/mL of DNS-LZM were used in this study. Fig. 4b shows the Fl-intensity of DNS-LZM in the presence of Re595 LPS. The intensity was also time- and temperature-dependent. Comparing s- and r-LPS, the Fl-intensities at 70°C were different. As shown in Fig. 5, the binding of 0111 LPS reached a maximum at 55°C, but that of Re595 was still increasing even at 70°C. These differences were also observed in the previous[14] results of enzyme inhibition, where significant enzyme inhibition by Re595 LPS was shown above 50°C instead of at 37°C in the case of 0111 LPS, and results from the differences of the physicochemical properties of micelles of each LPS.

To examine the binding more precisely, as the binding seems to be multiple and apparently involves irreversible processes, Scatchard analysis was performed to confirm the temperature dependence of the binding force. To estimate affinities, it is hypothesized that the Fl-intensity of the reaction mixture has a linear relationship to the quantity of LPS bound to DNS-LZM. Using this estimation, the percentage of LPS-bound DNS-LZM was calculated and affinities were estimated from the Scatchard analysis. Fig. 6 summarizes the percentage of DNS-LZM binding at various ratios and

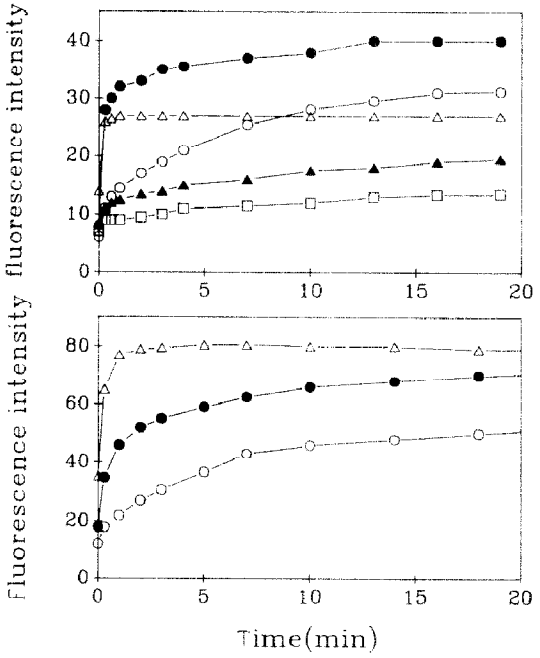

Fig. 4. Temperature dependence of the Fl-intensity of complex. [(*a*) (*upper*) Kinetics of formation of the 0111 LPS-DNS-LZM complex at various temperatures. DNS-LZM (5 μg) and 0111 LPS (5 μg) were mixed in 1.15 mL of PBS at various temperatures and the Fl-intensity (emi 490 nm) was monitored for 20 min at the indicated temperatures. □, 10°C; ▲, 25°C; ○, 37°C; ●, 50°C; △, 70°C. (*b*) (*lower*) Kinetics of formation of the Re595 LPS-DNS-LZM complex at various temperatures. DNS-LZM (5 μg) and Re595 LPS (5 μg) were mixed in 1.15 mL of PBS at various temperatures and the Fl-intensity (490 nm) was monitored for 20 min at the indicated temperatures. ○, 37°C; ●, 50°C; △, 70°C].

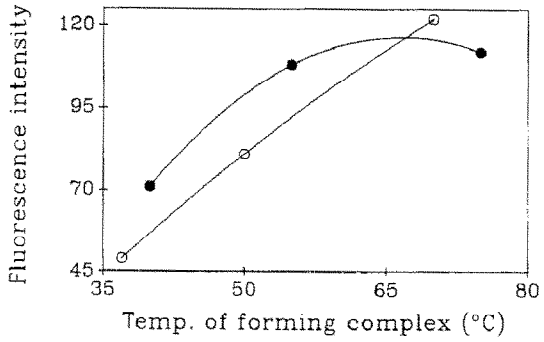

Fig. 5. Stability of the LPS-DNS-LZM complex by lowering temperatures. [The LPS-DNS-LZM complexes (5 μg LPS and 5 μg DNS-LZM in 1.15 mL PBS) were prepared at various temperatures. The Fl-intensity of each complex was measured at 25° after 40 min of incubation at 25°. ●, 0111 LPS; ○, Re595 LPS].

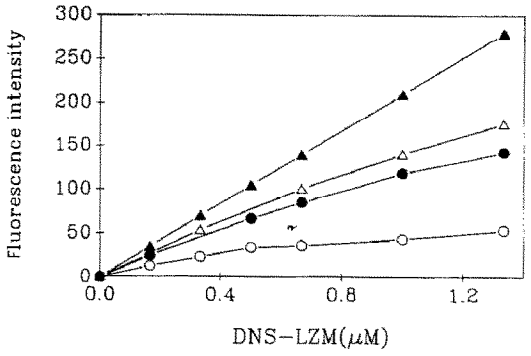

Fig. 6. Quantitative analysis of binding of DNS-LZM to LPS. [The ratios of DNS-LZM binding to LPS at various concentrations of DNS-LZM were measured. Binding of 100% was calculated from the Fl-intensity of DNS-LZM in the presence of an excess of LPS at each temperature. Values obtained at different temperatures were normalized to the relative Fl-intensity of those showing 100% binding. Dissociation constants for the complexes at various reaction temperatures were calculated by estimating quantities of bound and free forms of DNS-LZM from the Fl-intensity. See in the experimental section. ○, 25°; ●, 37°; △, 50°; ▲, 100% binding].

at various temperatures. Theoretical values (100% binding) of the Fl-intensity at each temperature are different, and thus Fig. 6 was prepared after normalizing each value. Calculated from this Figure by using the equation described in Materials and Methods, it is suggested that the dissociation constant of 0111 LPS and DNS-LZM is be 0.1 (25°), 0.08 (37°), and 0.02 (50°) μM^{-1}. These results also support the foregoing suggestions that the binding is temperature-dependent. As already described, the binding involves irreversible processes, as the complex, once produced at higher temperature, retained a higher Fl-intensity at lower temperatures. These results also suggest the contribution of multiple residues in both LPS and LZM toward the complex formation.

Ionic strength and pH dependence of the binding of LPS and LZM, as assessed by the fluorescence intensity of DNS-LZM. — In the previous study, we found that the

enzymic activity of LZM was inhibited by LPS and the inhibition was more significant[13] at acidic pH[1]. To determine the correlation between Fl-intensity and enzyme inhibition, the Fl-intensity of the LPS-DNS-LZM complex at various pH values and ionic strengths were examined. Fig. 7 shows the Fl-intensity of the complex prepared in various concentration of NaCl. The Fl-intensity was the strongest at the lowest ionic strength and gradually decreased to 0.5M NaCl, and only very weak fluorescence was observed above 0.5M NaCl, suggesting that the complex cannot be produced above 0.5M NaCl. As described in the previous section, the complex, once produced is relatively stable and this was not dissociated by dilution. To determine the stability of the complex at higher salt concentrations, the complex was added to various concentrations of NaCl solution and the Fl-intensities were compared. The Fl-intensity of the complex immediately after addition of various concentrations of NaCl was strongly dependent on the ionic strength, thus 62 (0M), 52 (0.075M), 50 (0.15M), 45 (1.1M), and 42 (2M), suggesting a dependence, at least in part, of the Fl-intensity to the ionic strength of the environment. However, even in 2M NaCl, the Fl-intensity was stronger than that produced in 2M NaCl, as shown in Fig. 7, and was only slightly changed over 15 min (Fig. 7, *inset*). It is suggested that the complex is also stable over a wide range of salt concentrations. Inhibition of the enzymic activity of LZM by LPS is modified by various compounds, such as, detergents, the antibiotic polymyxin B, NaCl, and by disialoganglioside. In the case of NaCl, increasing the NaCl concentration ($\sim$0.25M) also decreased the inhibitory effect of LPS. Considering these facts, the enzyme inhibition and Fl-increase by LPS appear to be closely related phenomena. The available evidence suggests that ionic as well as hydrophobic interactions between LZM and LPS are important for complex formation, and thus NaCl affects the ionic interaction of LZM and LPS.

As already described, enzyme inhibition by LPS is dependent on pH. The Fl-intensity of the complex over various pH ranges were measured by using three buffer systems, Tris (0.1M), acetate (0.1M), and phosphate-buffered saline (Fig. 8). Interestingly, the Fl-intensity was significantly dependent on pH and the buffer used. Higher Fl-intensities were observed at high pH in all three buffer systems, and the pH dependency was high in acetate and Tris and low in phosphate. In contrast to the enzyme inhibition, especially at pH 4 in acetate buffer, only a slight increment of the Fl-intensity was observed, in contrast to that at the other reaction pH values. As the enzymic activity of LZM was significantly inhibited at pH 4, a pH dependence of the Fl-intensity would exhibit negative correlation with the enzyme inhibition. Considering these facts, it is suggested that there are at least two kinds of binding sites on LZM, one being strongly related to Fl-intensity and the others to enzyme inhibition.

To determine the stability of the complex at various pH values, the complex was prepared at pH 7 and diluted under various conditions as shown in Fig. 8 (*inset a, b*). As in the case of ionic strength, the complex was very stable under these conditions and showed significant Fl-intensity at pH 4 during 15 min at 37°.

Specificity of the fluorescence of LZM-LPS complex. — The LPS-LZM complex is physiologically important because, for example, addition of LPS to such biological fluids as serum, saliva, and milk, inhibits LZM activity[13]. To examine the specificity of

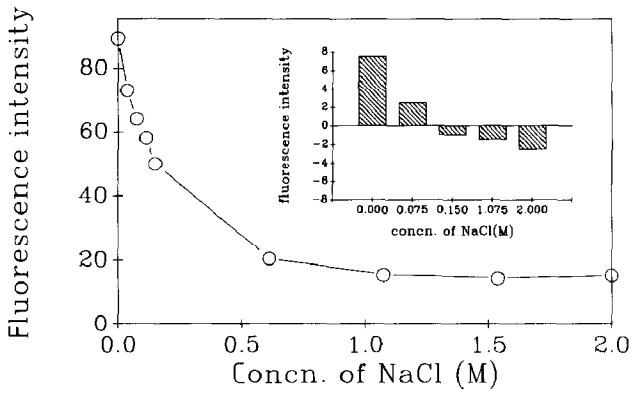

Fig. 7. Effect of NaCl concentrations on the Fl-intensity of complex. [DNS-LZM (5 μg) was added to PBS (1 mL) containing 0–2M NaCl. The 0111 LPS (50 μg) was added, the mixture incubated for 15 min at 37°, and the Fl-intensity was measured. The *inset* shows the stability of the complex in various concentration of NaCl. DNS-LZM (100 μL, 50 μg/mL) was added to 50 μL of 0111 LPS (100 μg/mL) and incubated for 40 min at 37°. Each complex was then added to 1 mL of PBS containing various concentration of NaCl. The Fl-intensity was measured immedately after mixing (Fl-0) and after incubation for 15 min at 37° (Fl-15). Changes of the Fl-intensities during 15 min (Fl-15)–(Fl-0), are indicated as each bar].

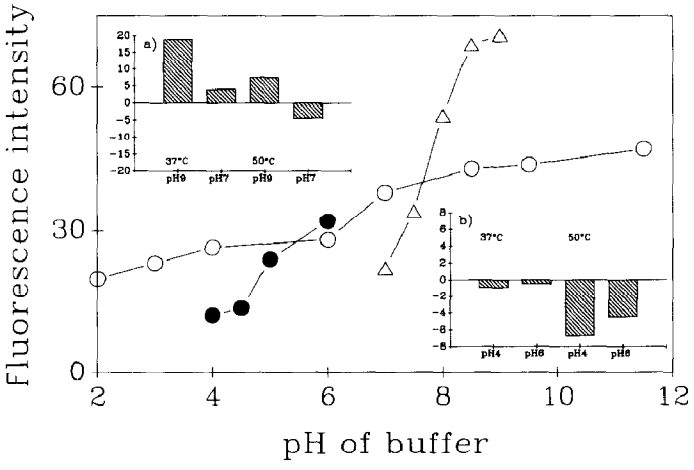

Fig. 8. Effect of pH on the Fl-intensity of the complex. [DNS-LZM (5 μg) was added to 1 mL of PBS, 0.1M acetate buffer, or 0.1M Tris-HCl buffer at various pH values. The 0111 LPS (50 μl, 100 μg/mL) was added, the mixture was incubated for 15 min at 37°, and the Fl-intensity was measured. ○, PBS; ●, acetate; △, Tris. *Insets* (*a*) and (*b*) show the stability of the complex in acetate buffer (*a*) and in Tris buffer (*b*), respectively. DNS-LZM (100 μL, 50 μg/mL) was added to 50 μL of 0111 LPS (100 μg/mL) and the mixture was incubated for 40 min at 37°. Each complex was then added to 1 mL of acetate (or Tris) buffers having various pH values. The Fl-intensity was measured immedately after mixing (Fl-0) and after incubation for 15 min at 37° (or 50°) (Fl-15). Changes of the Fl-intensities during 15 min (Fl-15)–(Fl-0) are indicated as each bar].

binding between LPS and LZM, the Fl-intensities of other DNS-derivatives and amphiphiles were compared.

The first experiment was designed to use several detergents listed in Table I instead of LPS, and the Fl-intensities were compared. Fig. 2 shows a representative fluorescence profile between DNS-LZM and Cetavlon (**7**). The Fl-intensity of DNS-LZM was significantly increased as low as 20 μg/mL in the solution and reached a maximum at a concentration of 100 μg/mL (Fig. 9a). The maximum Fl-intensities caused by Cetavlon were significantly stronger than those caused by LPS. The results with derivatives are summarized in Table I. Significant Fl-intensities were observed with CHAPSO (**14**), CHAPS (**13**), SDS (**3**), Cetavlon (**7**), and cardiolipin (**15**), but not with sodium deoxycholate (**4**), Triton X-100 (**9**), NP-40 (**10**), and *n*-octyl β-D-glucopyranoside **12**.

To determine the specificity of DNS-LZM, some DNS-derivatives were used instead of DNS-LZM. The fluorescence intensity of DNS-polymyxin B was also increased in the presence of LPS. However, none of the DNS-amino acids, basic or hydrophobic, showed an increase in Fl-intensity by LPS.

The Fl-intensities of various DNS-derivatives *vs.* detergents were also examined. Results are shown in Table I. As already described, the Fl-intensity of DNS-polymyxin B was increased by the addition of smooth as well as rough LPS. The Fl-intensities of DNS-polymyxin B were also increased by several detergents. The Fl-intensities for some of the detergents (especially Triton X100) could not be determined because of very high background. Interestingly, the Fl-intensity of DNS-polymyxin B was significantly increased by the addition of **2**, whereas the Fl-intensity of DNS-LZM was not increased. Furthermore, the Fl-intensities with Cetavlon and SDS were significantly lower than those observed in DNS-LZM. The Fl-intensities of DNS-amino acids were not increased by LPS, but some of the detergents, such as Cetavlon, SDS, and cardiolipin, increased the Fl-intensity (Table I). Dose responses of Fl-intensity of DNS-amino acids are shown in Fig. 9. Interestingly, DNS-derivatives of basic amino acids (cadaverine, arginine, and lysine) showed higher Fl-intensities in SDS, and those of hydrophobic amino acids (phenylalanine and leucine) showed higher Fl-intensities in Cetavlon. Considering these facts, the environment of the DNS-group appears to strongly influence the reactivity. It is noteworthy that the Fl-intensity of DNS-LZM was saturated at the lowest concentration of detergents used with both SDS and Cetavlon.

As already described, the binding of LZM to LPS was dependent on time as well as temperature, and higher temperatures induced a higher Fl-intensity over a shorter period of time. To compare the kinetics of binding between LPS and detergents to LZM, Cetavlon was added to DNS-LZM at various reaction temperatures. To examine the specificity, DNS-Leu was also used. As shown in Fig. 10, the maximum Fl-intensity was observed at 25–37° in the case of the DNS-LZM-Cetavlon complex. Furthermore, the DNS-Leu-Cetavlon complex was produced at lower temperature, soon after mixing.

Considering these facts, it is suggested that (*a*) increasing the Fl-intensities by dansylated derivatives is specific for substances having high affinity to LPS, because LZM and polymyxin B were the only substances to increase fluorescence, (*b*) DNS-

TABLE I

Effects of detergents on the fluorescence of DNS-derivatives[a]

Detergent	*Lysozyme*	*λ max (nm) 540*	*Leucine*	*λ max (nm) 555–560*	*Polymyxin*	*λ max (nm) 470–500*
1	+ + + + + + + + + +	505	.	560	?	?
2	.	525	.	560	+ + + +	500
3	+ + + + + + + + +	510	+ + + + + + +	540	+	520
4	.	540			.	460
6	.	485			?	?
7	+ + + + + + + + + +	510	+ + + + + + + + + + + + + + +	530	+ +	510
9[b]	?	?			?	?
10	+	525			?	?
12	.	535			?	?
13	+ + +	510			+ + +	470
14	+ + +	500	.	550	+ + + + +	490
15	+ + + + + + + + + +	500	+ + + +	540	?	?
Re595	+ +	500	.	555	+ + + + + + + + +	485
LPS0111	+ +	500	.	560	+ + + + + +	475

[a] + increase of Fl-intensity; . no change; ? unknown. [b] Difficult to measure the Fl-intensity because of high background.

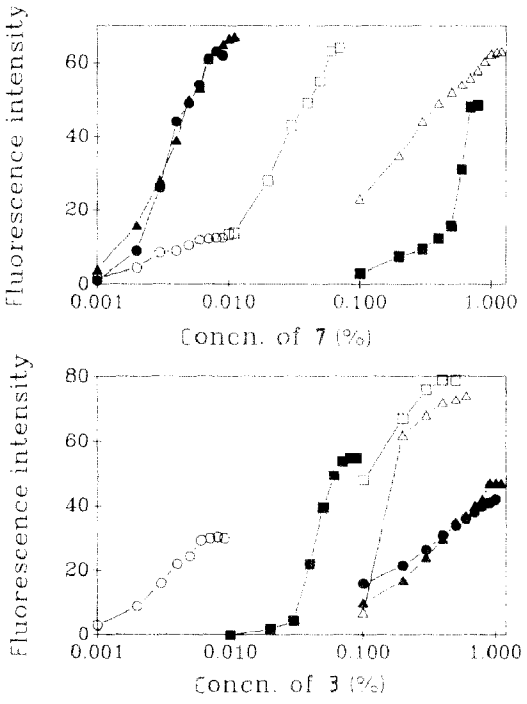

Fig. 9. Fl-intensity of various DNS-derivatives in amphiphiles. [(*a*) (*upper*) Fl-intensity of various DNS-derivatives in the presence of Cetavlon. DNS-LZM (100 μL, 50 μg/mL) or DNS-amino acids (1.5 μg/mL) were added to 1 mL of PBS. Cetavlon (**7**) was added to the concentrations indicated in the Figure and the Fl-intensity was measured. ○, LZM; ●, Phe; △, Arg; ▲, Leu; □, Lys; ■, cadaverine. (*b*) (*lower*) Fl-intensity of various DNS-derivatives in the presence of SDS. DNS-LZM (100 μL, 50 μg/mL) or DNS-amino acids (1.5 μg/mL) was added to 1 mL of PBS. SDS (**3**) was added to the concentrations indicated in the Fig. and Fl-intensity was measured. ○, LZM; ●, Phe; △, Arg; ▲, Leu; □, Lys; ■, cadaveline].

LZM as well as DNS-polymyxin B have their own specificities for increasing Fl-intensities according to the environment of the DNS-group, such as specific peptide-sequences, and (*c*) the amphiphilic property of LPS is important for fluorescence, and the kinetics of binding are dependent on the fluidity of amphiphiles as well as on the molecular weight of DNS-derivatives.

LZM enzymic activity in the presence of amphiphiles. — The inhibition of enzymic activity of LZM by both s- and r-LPS is dependent on time and temperature. The enzymic activity of LZM is also inhibited by several detergents as described in Table II, but not in exactly the same manner as detergents showing high Fl-intensities with DNS-LZM. For example, **4**, **12**, and **2** showed enzyme inhibition, but did not increase the Fl-intensity, whereas Cetavlon (**7**) did not inhibit enzymic activity but did increase the Fl-intensity. Considering these results, at least two kinds of interactions occur with LZM.

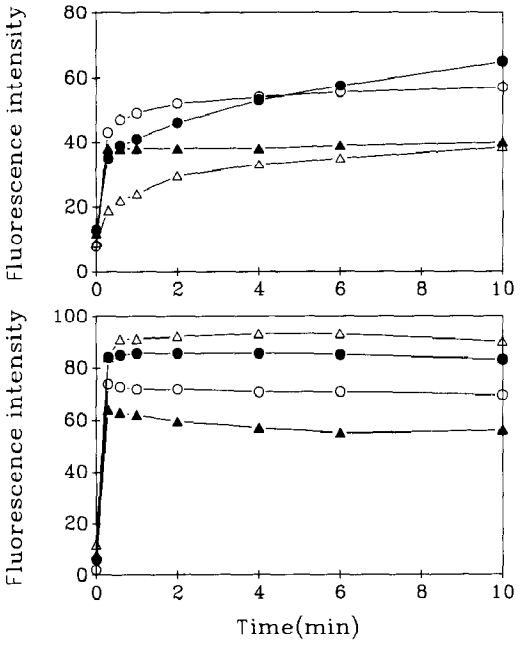

Fig. 10. Kinetics of the complex formation between Cetavlon and DNS-derivatives. [(*a*) (*upper*) Kinetics of formation of Cetavlon-DNS-LZM complex at various temperatures. DNS-LZM (100 μL, 50 μg/mL) was added to 1 mL of PBS and 100 μL of Cetavlon (1%) was added. The Fl-intensity (490 nm) was monitored for 10 min at various temperatures. △, 10°; ●, 25°; ○, 37°; ▲, 50°. (*b*) (*lower*) Kinetics of formation of Cetavlon-DNS-Leu complex at various temperatures. DNS-Leu (100 μL, 1.5 μg/mL) was added to 1 mL of PBS and 100 μL of Cetavlon (1%) was added. Fl-intensity (490 nm) was monitored for 10 min at various temperatures. △, 10°; ●, 25°; ○, 37°; ▲, 50°].

TABLE II

Effects of detergents on the enzymic activity of lysozyme[a]

	Detergent	
1	(1-sodium decanesulfonate)	+ + + + + + + +
2	(sodium octadecanoate)	+ + + + + + + +
3	(sodium dodecyl sulfate)	+ + + + + + + + + + +
4	(sodium deoxycholate)	+ + + + +
5	(lithium dodecyl sulfate)	+ + + + + + + + + + +
6	(cetylpyridinium chloride)	.
7	(Cetavlon)	.
8	(dodecyltrimethylammonium bromide)	.
9	(Triton X-100)	-----
10	(Nonidet P-40)	-----
11	(Tween20)	------
12	(*n*-octyl β-D-glucopyranoside)	+ + + + +
13	(3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)	+ + + + + +
14	(3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-propanesulfonate)	+ + + +
15	(Cardiolipin)	+ + + + + + + +

[a] + inhibition, - activation, . no effect.

DISCUSSION

The binding of LZM to LPS inhibits the biological activities of LPS as well as the enzymic activity of LZM. The purpose of this study was to clarify the characteristics of binding between LZM and LPS as precisely as possible. Dansylation of LZM is thought to modify only one Lys residue[16]. Increasing the Fl-intensity of the DNS-group appears to be mediated by the interaction or close association of the DNS-group with a certain part or parts of LPS molecule. Enzyme inhibition of LZM is also be mediated by the interaction of the active site with LPS. In the previous study, we found that the optimum ratio for inhibition of enzymic activity of LZM was ~20–25 μg 0111 LPS/1 μg LZM, suggesting that only some LZM molecules bind to one aggregated structure of 0111 LPS. The immunopharmacological activities of LPS are modified by LZM in the ratio of 1 μg Re 595 LPS/1–5 μg LZM. Interestingly, the Fl-intensity was saturated approximately in the ratio of 1 μg 0111 LPS/1 μg LZM. Considering these facts, several kinds of binding may occur between LZM and LPS. The following evidence also supports this concept. As described in this paper, binding of LPS to LZM is found to be time-dependent; the complex is gradually produced becomes stabilized within 20 min at 37°, 10 min at 50°, and 1 min at 70°. The maximum level of binding is also dependent on the reaction temperature, and more complex is formed at higher temperature. Complex formation is strongly dependent on the concentration of salt and complexation is not observed at >0.5M NaCl. However, the complex produced at lower salt concentration is stable at high salt concentration. It is also suggested that the complex formation depends on pH, with better complexation at higher pH. As with the salt stability, the complex was also stable at low pH. However, as discussed later, the pH dependence of complex formation was not positively correlated with enzyme inhibition.

From the collected evidence of the Fl-intensities of various DNS-derivatives and amphiphiles, it is concluded that LZM interacts with LPS by multiple binding-modes. The first is strongly related to the enzyme inhibition, the second is close to the Fl-intensity, and the third is dependent on the inhibition of immunopharmacological activities. However, it is possible that at least a part of each binding mode covers the same region of the molecules, and the exact binding sites related to each phenomenon are certainly not clarified yet. Additionally, the binding mode of Cetavlon was similar to the second one; sodium deoxycholate (**4**), **12**, and **2** were similar to the first, and SDS (**3**), CHAPS (**13**), CHAPSO (**14**), decansulfonic acid (**1**), and cardiolipin (**15**) include the first and the second modes of binding. The affinities of each mode were strongly dependent on pH: enzymic activity was strongly inhibited at lower pH, but the Fl-intensity was increased at higher pH. In addition, the kinetics of complex formation between LZM and LPS was also dependent on the reaction temperature; higher temperature required shorter times for saturation. The kinetics were also controlled both by the fluidities of LPS and LZM, because the kinetics of the LZM-Cetavlon complex and the DNS-Leu-Cetavlon complex were saturated faster than the LZM-LPS complex.

LZM is known to show fusogenic properties and has the capability for insertion into micelles[17,18]. In these micelles, the LZM molecule is protected from the proteolytic digestion. LZM is also known to bind to cardiolipin, SDS, and phosphatidic acid, and LZM can also act in certain organic solvents[19–22]. All of these lines of evidence support the binding of LZM to LPS. In addition, we have studied the reactivity of the LZM-LPS complex with several anti-LZM monoclonal antibodies, HyHEL-5,8,9,10, and 11, and found that reactivity to antibodies against all of the domains were significantly decreased[12]. Considering these facts, the LZM molecule appears to be inserted relatively deeply into the LPS aggregate, and the dimensions of the inserted portion are modified by changing the reaction pH.

LZM is a hydrolytic enzyme of bacterial cell-walls and acts at the stage of initial, nonspecific self-defense at a variety of sites. The isoelectric point of LZM is very basic. Several antimicrobial proteins and peptides are found in a variety of organisms, and many of these have basic pI values. Polymyxin B, an antimicrobial antibiotic, has a basic pI and has similar properties to LZM, such as binding to micelles and inhibition of the biological activities[23,24] of LPS. Myelin basic protein also inhibits the immunopharmacological activities of LPS and has the ability to interact with biological membranes[25,26]. The mechanisms of action of each of these antimicrobial substances appear to be individual but similarities in their biochemical and biophysical properties permitted us to presume some similarities also in their mechanisms of action.

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