第 2 章

スパイク H タンパク質の認識にリポ多糖の脂質部分の寄与

本章に関する研究内容は，雑誌論文に公表した。
以下にその論文を収録した。

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Crucial role of the lipid part of lipopolysaccharide for conformational change of minor spike H protein of bacteriophage ϕX174.

Minoru Inagaki,* Hirohito Wakashima, Muneharu Kato, Koji Kaitani, Shiro Nishikawa.

Department of Lifescience, Faculty of Bioresources, Mie University, 1577 Kurima-machiya, Tsu, Mie 514-8507, Japan.

Keywords: Bacteriophage ϕX174; Minor spike H protein; Lipopolysaccharide; Host-recognition; Conformational change; Phage infection

*Corresponding author. Tel: +81-59-231-9618; Fax: +81-59-231-9684.
E-mail address: inagaki@bio.mie-u.ac.jp (M. Inagaki).

Abstract

The contribution of the lipid part of lipopolysaccharide (LPS) to recognition by minor spike H protein of bacteriophage ϕX174 was investigated by comparing the interactions of H protein with LPS and its deacylated derivatives. The fluorescence and circular dichroism (CD) spectra of H protein increased upon binding to intact LPS and a partially deacylated derivative. In contrast, completely deacylated derivatives showed lower affinities and almost no fluorescence or CD changes of H protein. These results demonstrate that the lipid part of LPS is responsible for the conformational change of minor spike H protein, which would function as a trigger for phage DNA ejection for infection of the host cell.

1. Introduction

Bacteriophage *ϕX174* is a small icosahedral virus that consists of a single-stranded circular DNA and four capsid proteins, designated F, G, H and J [1]. The phage has a spike protrusion composed of five major spike G proteins and one minor spike H protein [2] at each of its icosahedral vertices, and adsorbs onto host enterobacteria, such as *Escherichia coli* C, through these spikes [3]. Thus, the spike proteins have been considered to recognize lipopolysaccharide (LPS), a major component of the outer membrane of enterobacteria, as a receptor for infection. We previously prepared the G [4] and H [5] proteins as fusion proteins with a hexa-histidine tag, and revealed their specific interactions with the LPS of *E. coli* C as the host strain. The interactions of the G [6] and H [7] proteins with LPS were analyzed quantitatively by their fluorescence spectra in order to determine their binding parameters. When H protein binds with LPS, the intensity of its fluorescence increases and its maximum emission wavelength (l_{max}) is blue-shifted [7]. These observations indicate that H protein changes its conformation upon interacting with LPS. H protein is thought to play a role in phage DNA ejection into the bacterial cell, a process known as 'eclipse', by penetrating the bacterial membrane after conformational changes of the capsid proteins [8]. The conformational change in H protein is now an attractive candidate for a switch that triggers DNA ejection. In this study, the LPS of *E. coli* C was subjected to limited chemical degradations to remove some or all of its fatty acid chains, and the interactions of the degraded LPS derivatives with H protein were analyzed by fluorescence and circular dichroism (CD) spectra, in order to determine which parts of LPS affect the conformational change of H protein.

2. Materials and methods

2.1. Materials

The LPS of *E. coli* C was extracted from cultured cells [9] by the phenol-chloroform-petroleum ether method [10]. Hexa-histidine-tagged H protein was obtained according to previously described methods [5,7], and its concentration was determined from the absorbance at 280 nm by Edelhoch's equation

[11].

2.2. Limited chemical degradations of LPS

The LPS of *E. coli* C was treated with anhydrous hydrazine (NH_2NH_2) at 37°C for 30 min [6] to form *O*-deacylated LPS (deO) and then further treated with 4 M KOH at 125°C for 18 h [12] to form *O, N*-deacylated LPS (deON). The polysaccharide part of LPS (PS) was obtained by hydrolyzing the LPS of *E. coli* C in 1% acetic acid at 100°C for 2 h [6,13]. These degraded LPS derivatives were purified by gel filtration through Bio Gel P4 in a 1.5 cm f × 110 cm column (Bio-Rad, Hercules, CA, USA) eluted with pyridine:acetic acid:H_2O = 10:5:1000 (v/v/v) at pH 4.2. The saccharide-containing fractions were detected using a phenol-H_2SO_4 coloring technique [14], combined and lyophilized.

2.3. Fluorescence spectra of H protein in the presence of LPS and its derivatives

The fluorescence spectra of H protein were measured using a Shimadzu RF5300PC spectrometer. The excitation wavelength (280 nm), excitation slit width (±1.5 nm) and emission slit width (±15 nm) were fixed. Each of the ligands (0.69 mM) in 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl was added to an aliquot of H protein (0.33 μM) in the same buffer in a quartz cell at 25°C using a microsyringe. The mixtures were then incubated for 2 min, and the fluorescence spectra (300-500 nm) were recorded at a scan rate of 10 nm/min.

2.4. Fluorometric titrations of H protein with LPS and its derivatives

Fluorometric titrations of H protein with LPS and its derivatives were performed using a Hitachi 650-560 spectrometer. The excitation wavelength (280 nm) and excitation slit width (±1 nm) were fixed throughout the experiments. Each of the ligands (0.69 mM) in Tris-buffered saline (TBS; 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl) was added to an aliquot of H protein (0.49 μM) in the same buffer in a quartz cell at 25°C using a microsyringe. The fluorescence intensity changes after the addition of the ligands were monitored at emission wavelengths of 326±12 nm for LPS and deO and 354±12 nm for deON and PS. The intensities were corrected by the dilution factor caused by the addition of each ligand solution, and calculated as the relative increase or decrease (in %) based on the intensity in the absence of the ligand solution (set as 100%).

2.5. CD spectra of H protein in presence of LPS and its derivatives

CD spectra (200-250 nm) of H protein (5.0 μM) in 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl were recorded in the presence of LPS (10 μM) or deO, deON or PS (80 μM each) using a JASCO J-720M spectropolarimeter and the software provided by the manufacturer. The scanning parameters were: sensitivity: 20 mdeg; scan speed: 10 nm/min; band widths: 1.0 nm. The measurements were carried out at 25°C in a quartz cell (1 mm).

3. Results

3.1. Changes in the fluorescence spectra of H protein in the presence of LPS and its derivatives.

The LPS of *E. coli* C was subjected to limited chemical degradations using anhydrous hydrazine, 4 M KOH or 1% acetic acid to remove some or all of the fatty acid chains attached to the lipid A part of LPS, according to previously reported procedures [6,12,13] (Fig. 1). The obtained derivatives, namely *O*-deacylated LPS (deO), *O, N*-deacylated LPS (deON) and the polysaccharide portion of LPS (PS), were each purified by gel filtration, and then crosschecked for their integrity by chemical and biochemical methods [6].

Fluorescence spectra of H protein were recorded in the absence or presence of LPS and its derivatives (Fig. 2). H protein showed a bell-shaped fluorescence emission spectrum upon excitation at 280 nm. The maximum emission wavelength, l_{max}, was observed at 354 nm. Since this l_{max} was in such a high field that it was similar to that of a tryptophan solution in water, the tryptophan residues in H protein may be significantly exposed to the solvent water. Following the addition of LPS (Fig. 2A), the fluorescence intensity around 300-360 nm increased dose-dependently. The emission peak, l_{max}, shifted toward a shorter

wavelength, indicating a hydrophobic interaction between the tryptophan residues of H protein and LPS. An increased intensity and a l_{max} shift were also observed in the presence of the deO derivative (Fig. 2B), although the degrees of the changes were moderate compared with those after LPS addition. On the other hand, no increases in the fluorescence intensity of H protein or l_{max} shifts were observed in the presence of the deON and PS derivatives (Fig. 2C and 2D). Since these latter derivatives had lost the hydrophobic fatty acid chains, their addition did not provide H protein with a hydrophobic environment, and therefore the tryptophan residues of H protein showed reduced fluorescence intensity.

The fluorescence difference spectra of H protein during the addition of LPS and its derivatives are shown in Fig. 3. The difference spectra for LPS (Fig. 3A) and deO (Fig. 3B) showed a major maximum at 325 nm and a major minimum at 380 nm. The isosbestic points were observed at 360 and 426 nm for LPS and 360 and 445 nm for deO. The difference spectra for deON (Fig. 3C) and PS (Fig. 3D) showed a major minimum at 350 nm and a major maximum around 452 nm for deON and 470 nm for PS. The isosbestic points were observed at 416 nm for deON and 437 nm for PS. All the difference spectra showed the same nature and only differed in their intensity during the addition of the ligands. These observations indicate that H protein was detected as a mixture of the free and bound states. Thus, free H protein has a l_{max} at 354 nm, H protein bound with LPS or deO has a l_{max} at 326 nm, and H protein bound with deON or PS has a l_{max} at 350 nm. These fluorescence spectra gradually changed according to the adduct of the spectra of the free and bound H protein.

3.2. Fluorometric titrations of H protein with LPS and its derivatives

The fluorescence intensity changes at 326 nm for LPS and deO and 354 nm for deON and PS were monitored to analyze the interactions of H protein with these ligands. The fluorescence intensity increase or decrease, DF (%), was calculated as a percentage relative to the intensity of H protein in the absence of a ligand, and plotted against the concentration of each ligand, $[\text{Ligand}]_0$ (μM), as shown in Fig. 4. Mean data points obtained from more than three independent experiments were applied to non-linear least square fitting to Eq. 1 by the Levenberg-Marquardt algorithm [15] provided by the PROFIT software from Quantum Soft Co. (Zürich, Switzerland).

$$\Delta F = \frac{\Delta F_{max} \times [\text{Ligand}]_0^{\,n}}{K_d + [\text{Ligand}]_0^{\,n}} \qquad \text{Eq. 1}$$

As unknown values, the dissociation constant, K_d (μM), of complexes of H protein with each ligand and the maximum fluorescence change, DF_{max} (%), which should be observed when all the H protein molecules are complexed with the ligand, were calculated using the initial concentration of each ligand, $[\text{Ligand}]_0$ (μM), and the change in the fluorescence intensity, DF (%), as known values. The solid lines in Fig. 4 represent the theoretical curves drawn according to Eq. 1 using the values listed in Table 1.

The K_d and DF_{max} values for LPS were calculated from Eq. 1, and revealed that the optimal binding was 10.9±0.8 μM and 77.4±2.2%, respectively. The parameters for the other ligands were also calculated and are listed in Table 1. The K_d and DF_{max} values for deO were 28.1±3.6 μM and 29.6±0.5%, respectively. The optimal n value (stoichiometry of binding) for the deO derivative was found to be 1.9±0.1. Fitting toward an S-shaped curve for titration with deO could be possible by introducing the assumption that H protein could accommodate more than one deO molecule. On the other hand, the n values for the deON and PS derivatives were settled at 1.0 without difficulty. The DF_{max} values for these two derivatives were -18.7±1.4% and -15.0±1.8%, respectively. The K_d values were approximately 35 μM for both derivatives. Although the change in fluorescence was drastically altered when the fatty acid chains of LPS were removed, the magnitudes of the affinities of the deON and PS derivatives were similar that of the deO derivative. When the hydrophobic fatty acid chains of LPS were removed, the affinity for H protein was decreased to one-third (31-39%) based on the affinity of intact LPS as 100%. Thus, the strong affinity for binding to H protein was attributable to the lipid part of LPS.

3.3. Changes in the CD spectra of H protein in the presence of LPS and its derivatives

The observed changes in the fluorescence spectra indicated that the conformation of H protein was altered upon interaction with LPS and its derivatives. Therefore, the CD spectra of H protein were monitored in the presence of the four kinds of ligands (Fig. 5).

The X-ray structure of H protein was not determined due to its dispersed electron density [16].

Part of H protein is thought to reside in a space formed by pentamers of the capsid F and spike G proteins and to interact with the DNA-binding J protein and phage DNA [17, 18]. The CD spectrum of the histidine-tagged H protein revealed that H protein has an a-helical secondary structure with negative peaks at 208 and 222 nm (Fig. 5A) [19]. When LPS or deO were added, the conformation of H protein changed in the direction for augmenting the signals of a-helices. In contrast, deON and PS induced conformational changes of H protein in the direction for decreasing the signals of a-helices. The CD spectra, as well as the fluorescence spectra, clearly demonstrated that LPS and its derivatives could be divided into two types, namely one type that increased both the fluorescence intensity and CD intensity and another type that decreased both the fluorescence intensity and CD intensity. The conformational changes induced by deON and PS were smaller than those induced by LPS and deO. These observations were also consistent with the findings that the absolute magnitudes of the fluorescence changes, DF_{max}, of deON and PS were smaller than those of LPS and deO. The quality of the interaction between H protein and LPS was drastically altered when the hydrophobic fatty acids were truncated.

4. Discussion

4.1. The lipid part of LPS plays a positive role in recognition by H protein

Comparisons of the interactions of H protein with intact LPS and its deacylated derivatives clearly revealed differences among the four kinds of derivatives, as evaluated by two different methods, namely the fluorescence and CD spectra. As a result, the recognition of LPS by spike H protein is considered to have two aspects: binding and conformational change. In a previous paper, we reported that different LPSs containing a common lipid A region but different R-core polysaccharide regions showed similar large increases in fluorescence intensity with different affinities [7]. The results of the present study confirmed that the large increase in fluorescence intensity was lost when the lipid region was truncated from LPS. Thus, we can now conclude that the lipid region of LPS is responsible for the drastic conformational change of H protein.

The conformational change undergone by H protein in response to LPS is considered to create a suitable structure for binding to LPS and subsequently switch on the DNA ejection process. If this conformational change in the direction for increasing the a-helix content, induced by intact LPS as well as the deO derivative, is identified as a "favorable conformational change", then, in sharp contrast, the conformational change in the direction for decreasing the a-helix content, induced by the deON and PS derivatives, must be considered to be an "unfavorable conformational change". Such an unfavorable conformational change is equivalent to the situation of non-productive binding of a substrate to an enzyme, in which binding occurs but the reaction does not. It is noteworthy that the lipid part of LPS performs not only a passive role as an anchor for the polysaccharide part onto the bacterial membrane but also a positive role as a trigger for the conformational change of H protein of bacteriophage ϕX174 .

4.2. Putative scenario for recognition of LPS by spike proteins for DNA ejection

The major spike G protein, which is a co-component of the spike structure with H protein, exists as a pentamer on each vertex of the icosahedral capsid in order to provide a hydrophilic channel for DNA ejection [8, 16-18]. G protein has also been shown to bind specifically with the LPS of *E. coli* C, reduce its fluorescence intensity and undergo a conformational change in the direction for increasing the CD signal intensity of b-sheet secondary structures [6]. This type of conformational change was also identified as a favorable conformational change for LPS binding. Although the affinities of the deacylated derivatives, deO, deON and PS, for G protein were significantly lower than that of intact LPS, the direction of the conformational change remained favorable as long as the derivatives retained the full-length R-core polysaccharide sequence of *E. coli* C. On the other hand, a favorable conformational change did not occur for LPSs with a truncated R-core polysaccharide sequence, even when their lipid parts were intact. Therefore, both the H and G proteins have been proved to bind to LPS and undergo conformational changes. Thus, the conformational changes of these two spike proteins are considered to be one of the triggers for the DNA ejection process.

An intact LPS structure is important for strong binding by both these spike proteins. However, the lipid region is essential for the conformational change of H protein, but not for that of G protein. Moreover, the polysaccharide residues of LPS provide different contributions to the recognition by G and H proteins, since the outer R-core residues are significant for H protein, whereas the inner R-core residues are

significant for G protein [20]. It is quite reasonable that the selective binding and conformational changes of the H and G proteins are complementarily controlled by the contributions of the lipid and polysaccharide parts of LPS. Therefore, coordinated changes in the conformations of these two spike proteins, as a trigger for DNA ejection, are suggested to be one of the primary mechanisms for host selection by ϕX174 .

Previously, a whole particle of ϕX174 was reported to eject its phage DNA via irreversible conformational changes of its capsid proteins, involving disruption of the interactions between the proteins and the phage DNA, followed by reversible adsorption onto the surface of a host bacterial cell [21]. From our current results, a putative scenario for ϕX174 infection, focusing on the functions of the spike proteins, is: 1) the phage adsorbs onto the bacterial cell through the spike proteins; 2) the phage recognizes the adsorbed cell as a host through binding of the spike proteins to LPS; 3) if the complementary requirements of both spike proteins are satisfied, the phage induces conformational changes of the spike proteins to release the phage DNA for infection.

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Table 1
Parameters determined by fluorometric titrations of H protein with LPS and its derivatives.[a]

Ligand	n[b]	K_d (μM)	DF_{max} (%)	Affinity (%)
LPS	1	10.9±0.8	77.4±2.2	100
deO	1.9±0.1	28.1±3.6	29.6±0.5	39±5
deON	1	35.2±4.5	-18.7±1.4	31±4
PS	1	35.5±7.9	-15.0±1.8	31±7

Values represent the mean ± standard deviation. [a] The molecular weights of LPS and its derivatives were calculated based on the most plausible chemical structures (Fig. 1): LPS (4324), deO (3478), deON (3028) and PS (1904). [b] The n value means the stoichiometry of binding.

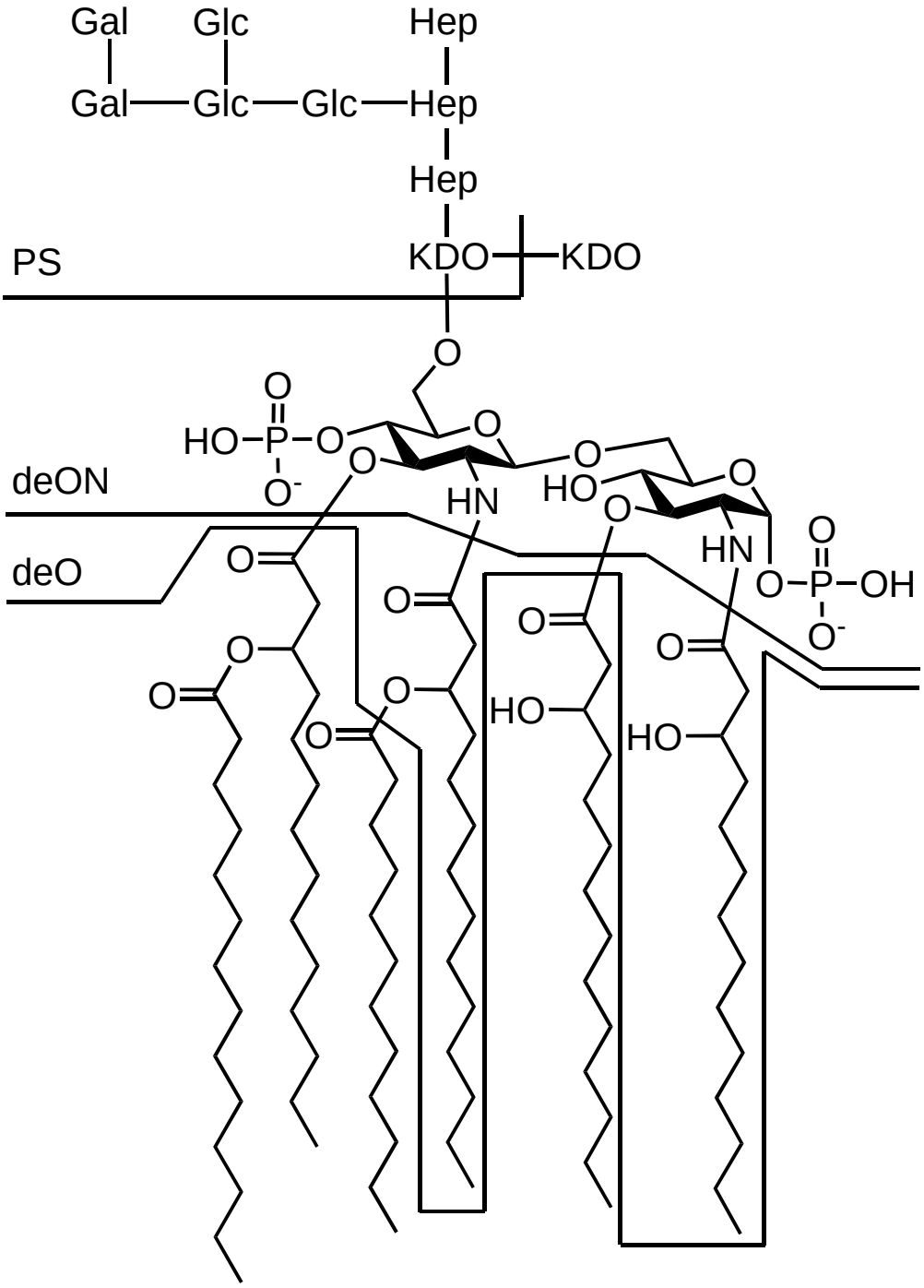

Fig. 1. Limited chemical degradations [6,12,13] of the LPS of *E. coli* C. Gal, D-galactose; Glc, D-glucose; Hep, L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-D-*manno*-2-octulosonic acid.

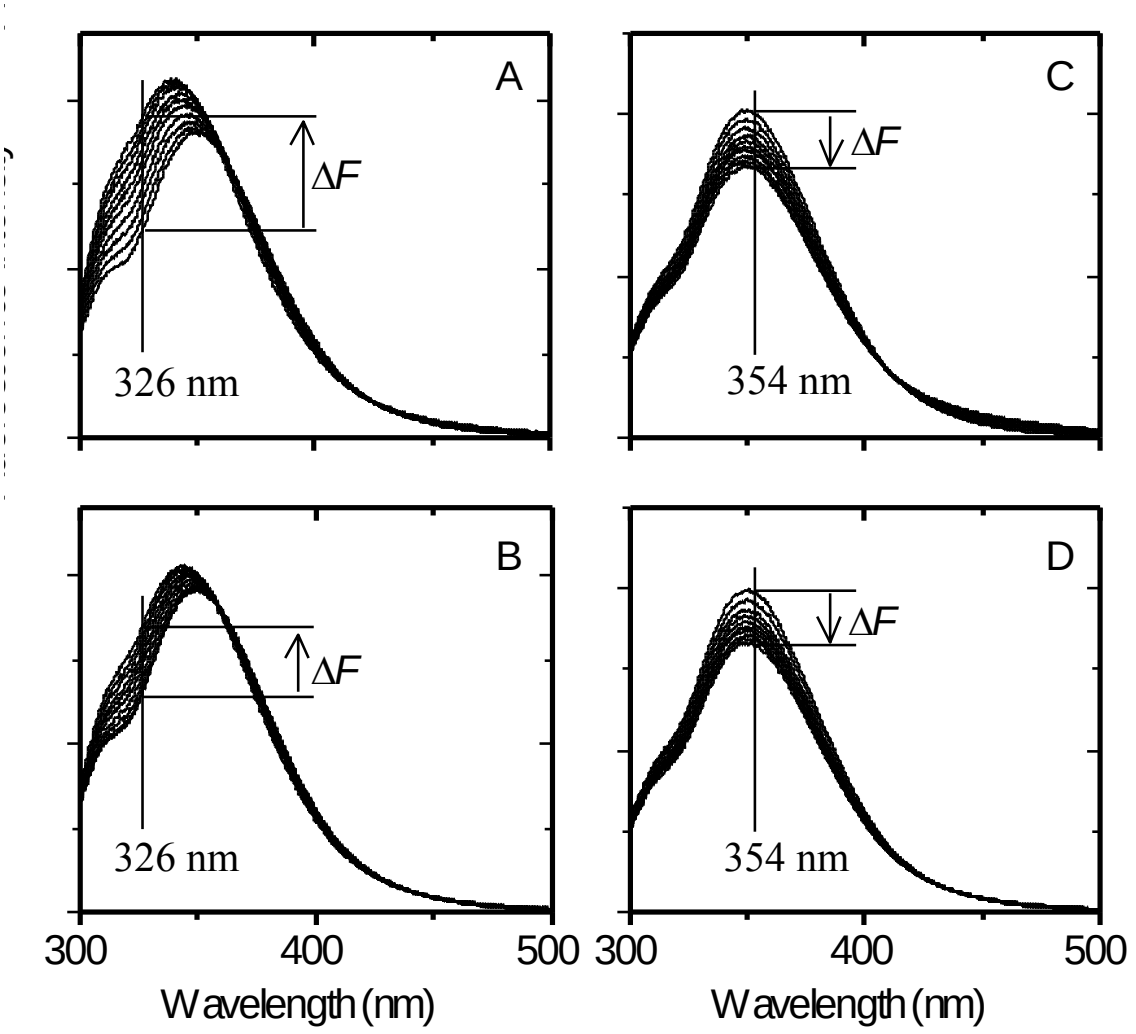

Fig. 2. Changes in the fluorescence spectra of H protein in the presence of LPS and its derivatives. Each ligand (0.69 mM) in 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl was added to a solution of H protein (0.33 μM) in the same buffer at 25°C. The final concentrations of LPS (A) and deO (B) were, from lower to upper, 0, 0.9, 1.7, 2.6, 3.4, 4.3, 5.1, 6.8, 8.5 and 11.9 μM. The final concentrations of deON (C) and PS (D) were, from upper to lower, 0, 3.8, 7.6, 11.3, 15.0, 18.7, 22.3, 25.8, 29.4 and 32.9 μM. All spectra were observed at an excitation wavelength, l_{ex}, of 280±1.5 nm and an emission wavelength, l_{em}, of 300-500±15 nm.

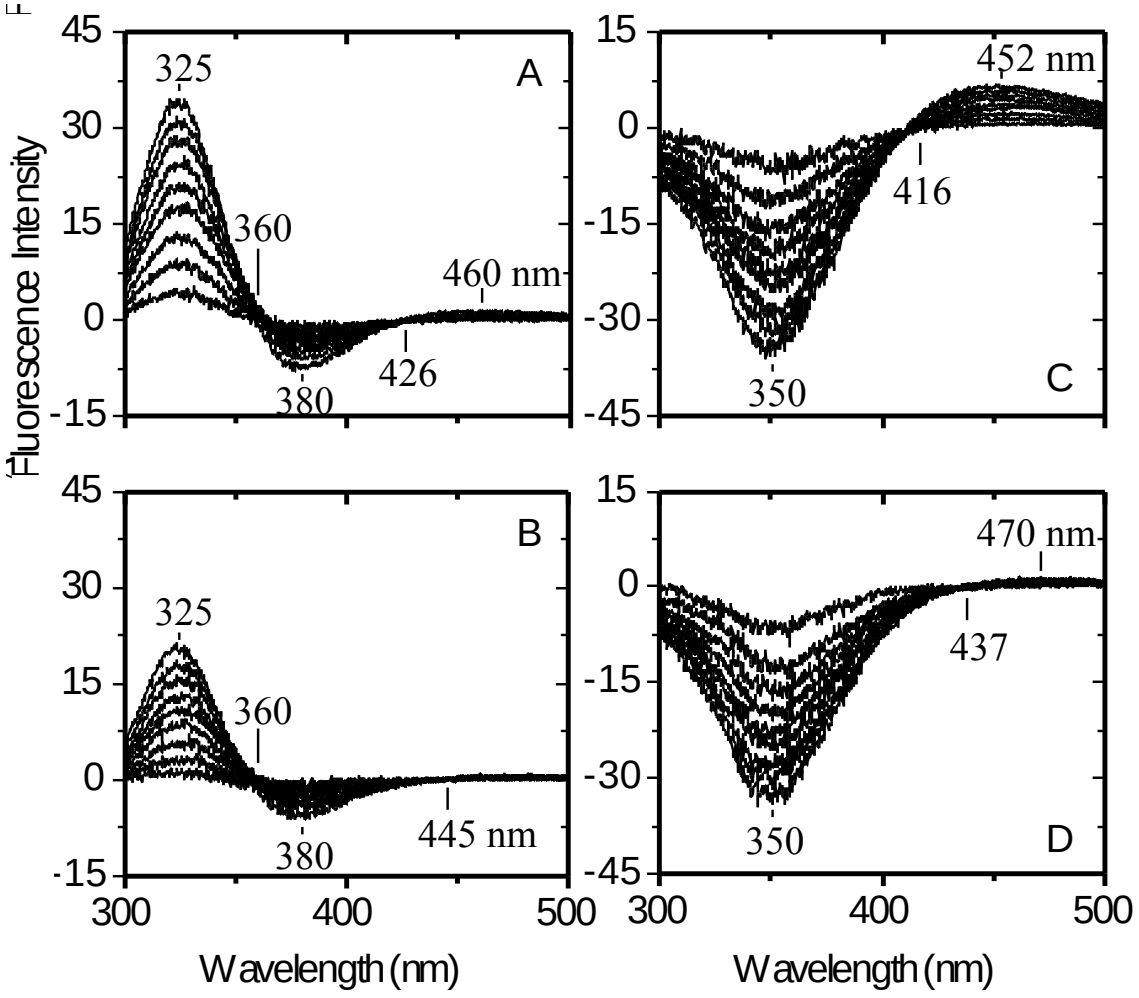

Fig. 3. Difference spectra of H protein in the presence of LPS and its derivatives. Based on the spectrum of H protein in the absence of a ligand, difference spectra were calculated based on the spectra shown in Fig. 2 after correction for the dilution factor. The final concentrations of LPS (A) and deO (B) were, from lower to upper, 0.9, 1.7, 2.6, 3.4, 4.3, 5.1, 6.8, 8.5 and 11.9 μM. The final concentrations of deON (C) and PS (D) were, from upper to lower, 3.8, 7.6, 11.3, 15.0, 18.7, 22.3, 25.8, 29.4 and 32.9 μM. The numbers indicate the wavelengths (nm) for the peaks and the isosbestic points.

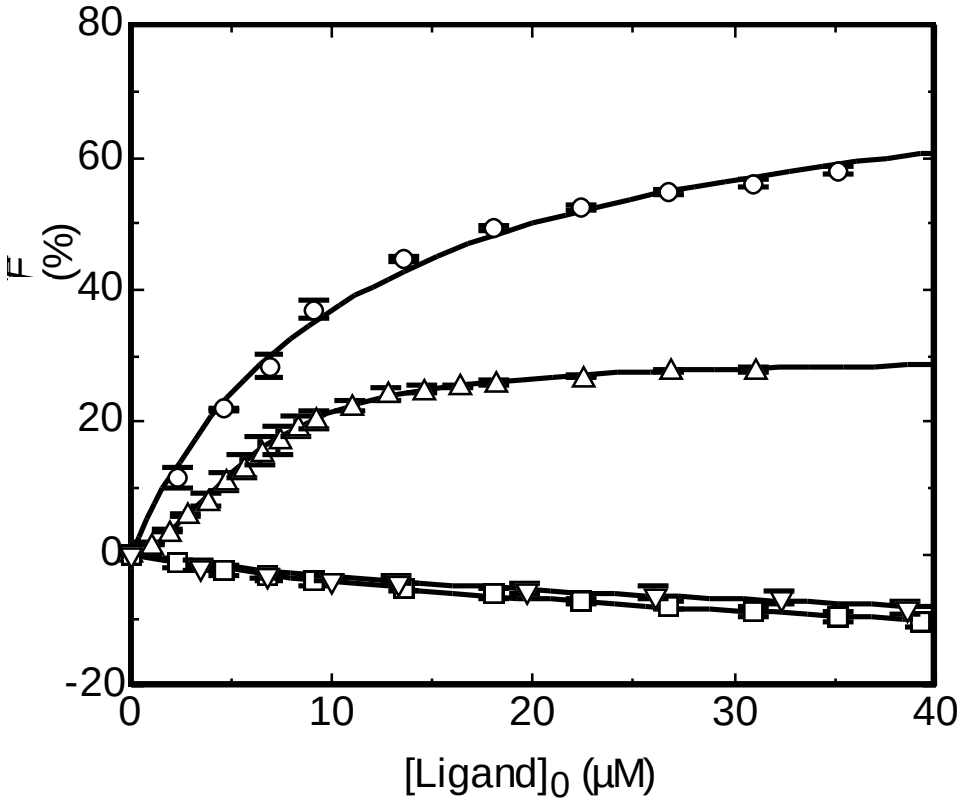

Fig. 4. Fluorometric titrations of H protein with LPS and its derivatives. The relative fluorescence intensity increases at 326 nm induced by LPS and deO and the relative fluorescence intensity decreases at 354 nm induced by deON and PS were monitored during the addition of the ligands. The relative fluorescence intensity changes were calculated as percentages, DF (%), based on the fluorescence intensity of H protein in the absence of a ligand, and plotted against the concentration of each ligand after correction for the dilution factor caused by addition of the ligand solution. The ligands added were: LPS (circles), deO (triangles), deON (squares) and PS (inverted triangles). Standard deviations at the 95% confidence level were calculated for more than three independent experiments. The K_d and DF_{max} values were obtained by non-linear least square fitting to Eq. 1. The solid lines in the Figure represent the theoretical curves drawn according to Eq. 1, using the calculated values listed in Table 1.

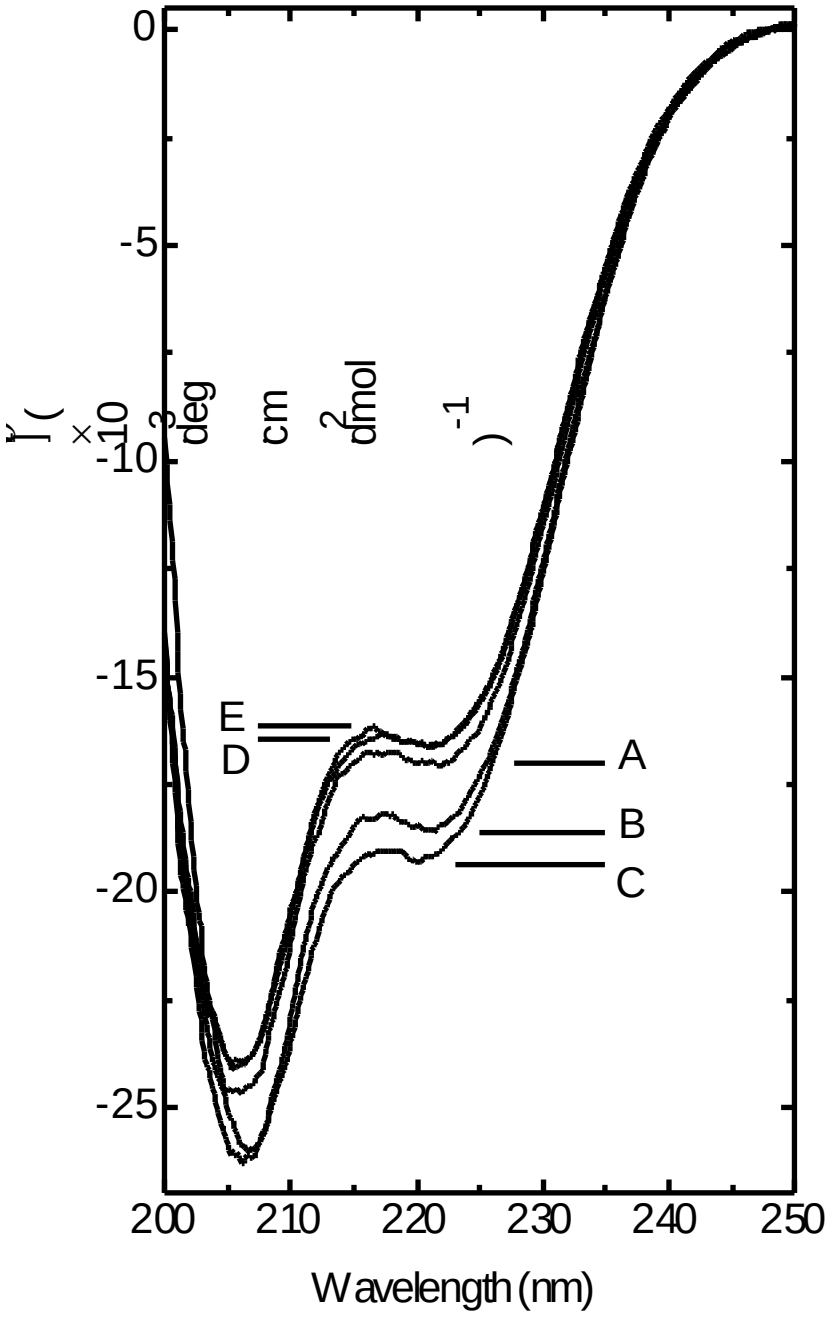

Fig. 5. Changes in the CD spectra of H protein in the presence of LPS and its derivatives. CD spectra of H protein (5 μM) were recorded in the absence of a ligand (A) and in the presence of 20 μM LPS (B), 80 μM deO (C), 80 μM deON (D) and 80 μM PS (E) in 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl at 25°C.