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Characterization of the Binding of Spike H Protein of Bacteriophage φX174 with Receptor Lipopolysaccharides[1]

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The spike H protein of bacteriophage φX174 was prepared as a hexa histidine-tagged fusion (HisH). On enzyme-linked plate assaying, HisH was found to bind specifically to the lipopolysaccharides (LPSs) of φX174-sensitive strains, *Escherichia coli* C and *Salmonella typhimurium* Ra chemotype, having the complete oligosaccharide sequence of the R-core on the LPSs. In sharp contrast, HisH bound weakly to the LPSs of φX174-insensitive strains, *i.e. E. coli* F583 (Rd_2) lacking some terminal saccharides and *E. coli* O111: B4 (smooth strain) having additional *O*-repeats on the R-core. The fluorescence spectra of HisH changed dose-dependently in the case of the LPS of *E. coli* C, the intensity increasing and the emission peak shifting to the shorter wavelength side, which was attributable to the hydrophobic interaction of HisH with the LPS. The binding equilibrium was analyzed by fluorometric titration to determine the dissociation constant K_d, 7.02 ± 0.37 μM, and the Gibbs free energy change ΔG^0, −29.1 kJ mol^{-1} (at 22°C, pH 7.4). Based on the temperature dependence of K_d in a van't Hoff plot, the standard enthalpy change ΔH^0 and the entropy change ΔS^0 were calculated to be +23.7 kJ mol^{-1} and 179 J mol^{-1} K^{-1} at 22°C, respectively, and this binding was thereby concluded to be an entropy-driven reaction.

Key words: bacteriophage φX174, binding analysis, lipopolysaccharide, receptor, spike H protein.

Bacteriophage φX174 is a small icosahedral virus with a single-stranded closed circular DNA of 5,386 bases and four capsid proteins, F, G, H, and J (*1*). φX174 recognizes lipopolysaccharides (LPSs), which are a major component of the outer membrane of Gram-negative bacteria, as receptors in the infection process (*2*). LPS comprises of three distinct components; *O*-repeats, R-core oligosaccharide, and lipid A. Of these three regions, the R-core oligosaccharide is supposed to be important for the recognition, because host bacterial strains for φX174 are limited to some rough mutants of *Enterobacteriaceae* having the complete sequences of the R-core oligosaccharide on their LPSs, such as *Escherichia coli* C, *Salmonella typhimurium* Ra chemotype, *Shigella sonnei* phase II, *etc.* (*3–5*).

Electron microscopic studies revealed that icosahedral-shaped φX174 became attached to the bacterial surface at the tip of one of twelve vertices, called spikes (*6*), and ejected its DNA through the spike to infect the host cell (*7*). Within the spike, which consists of five G proteins and one H protein (*8*), the H protein consisting of 328 amino acids is considered to recognize LPSs from several lines of indirect evidence: (i) host range mutations of φX174 were found in the H gene (*9*); (ii) an H protein-deficient phage could not infect host cells (*10*); (iii) when spikes were removed from phage particles by urea treatment (*8*), the spikeless capsids lost the ability of selective adsorption to host cells (*11*); (iv) the H protein–assisted transfection of phage DNA into spheroplast cells of φX174-insensitive strain was drastically inhibited by LPS of the sensitive strain and by anti-H serum (*12*). No study on the isolation or preparation of spike H as a single protein has been reported to date, and hence there is no direct evidence for the interaction of H with the receptor LPSs. Moreover, recent X-ray crystallog-

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[3] The data points for ΔF corresponding to the LPS in the low concentration range tended to shift slightly below the theoretical curve. Because the Hill's constant was minimized at *ca.* 1, binding stoichiometry (1:1) was assumed in this work. However, the possibility that more than one molecule of the LPS bound to HisH could not always be ruled out.

[4] A preliminary experiment involving the interaction of HisH with the deacylated LPS, which was prepared from the LPS by alkaline hydrolysis to remove hydrophobic fatty acids, showed that the fluorescence intensity of HisH did not increase but decreased on interaction with the deacylated LPS.

Abbreviations: Col, 3,6-dideoxy-L-galactose; ΔAbs_{max}, the maximum changes of absorbance; ΔF_{max}, the maximum fluorescence intensity change; Gal, D-galactose; Glc, D-glucose; GlcNAc, 2-acetamide-2-deoxy-D-glucose; Hep, L-*glycero*-D-*manno*-heptose; HisH, histidine-tagged H protein; IPTG, isopropyl β-D-thiogalactopyranoside; K_d, dissociation constant; KDO, 3-deoxy-D-*manno*-2-octulosonic acid; LPS, lipopolysaccharide; λ_{em}, emission wavelength; λ_{ex}, excitation wavelength; λ_{max}, peak wavelength of emission spectrum; P, phosphate residue; PEtN, 2-aminoethylphosphate residue; $S_{50\%}$, concentration needed for 50% saturation of the binding; STP-POD, streptavidine-peroxidase complex; TBS, Tris-HCl buffer saline.

raphy revealed the detailed structures of capsid proteins F, G, and J, however, the H protein remained invisible because of the diffused electron density (*13*). In the preceding study, we prepared the H protein as a hexa histidine–tagged fusion (HisH) and qualitatively showed its interaction with the LPSs of ϕX174-sensitive strains by means of an enzyme-linked plate assay involving a streptavidin-peroxidase complex as a detection tool (*14*).

In this study, the binding affinity of HisH toward a series of LPSs of ϕX174-sensitive and -insensitive strains was estimated by means of the enzyme-linked plate assay, and the binding equilibrium was quantitatively analyzed by fluorometric titration to determine the dissociation constants and thermodynamic parameters for HisH and LPSs for characterization of the driving force of the binding.

MATERIALS AND METHODS

Plasmid DNA preparation, electrophoresis of DNA fragments and other basic DNA manipulations were carried out by routine procedures (*15*). DNA sequencing was performed with an ABI 373S DNA sequencer using a Dye Termination Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA). N-Terminal amino acid sequencing was carried out with an ABI 476 sequencer (PE Applied Biosystems). Protein concentrations were determined by the Coomassie Brilliant Blue dye-binding method (Protein Assay Kit; Bio-Rad, Hercules, CA, USA).

Preparation of the Histidine–Tagged Spike H Protein (HisH)—The DNA fragment encoding the spike H protein was amplified by polymerase chain reaction (PCR) using *Ampli Taq* DNA polymerase (PE Applied Biosystems) and ϕX174 RF DNA (Toyobo, Osaka) as a template. The forward primer [5′-GCGGATCCGATGACGATGACAAAATGTTTG-GTGCTATTGCTGG-3′] had a *Bam*HI restriction site and the sequence for a proteolytic cleavage site for enterokinase. The reverse primer [5′-TTAAGCTTATTTCCTAGAC-AAATTAGA-3′] had a *Hin*dIII restriction site, which was located just behind the stop codon TAA. The PCR-amplified product was digested with *Bam*HI and *Hin*dIII, and the resultant fragment was ligated into the *Hin*dIII–*Bam*HI sites of pBluescript II KS(−) (Stratagene, La Jolla, CA, USA) to afford pBlue-H. After the DNA sequence of the inserted fragment had been confirmed, pBlue-H was digested with *Bam*HI and *Hin*dIII, and the resultant fragment (996 bp) was ligated into the *Bam*HI-*Hin*dIII sites of pQE-30 (Qiagen, Hilden, Germany) to yield the expression vector pQE-H. The expressed HisH in the cell lysate of *E. coli* M15 (pREP4) (Qiagen) harboring pQE-H was fractionated by affinity chromatography on Ni-NTA agarose according to the manufacturer's instructions (Qiagen). Further purification of HisH was accomplished by anion-exchange chromatography on DEAE-CELLULOFINE A-500 (Seikagaku Corp., Tokyo) to afford the purified HisH.

Lipopolysaccharides—The *E. coli* C strain was cultivated in 6 liters medium at pH 7.2 (*17*), and the LPS was extracted from the dried and defatted cells by the PCP method (*18*), the yield being 3.0% wt of bacterial dry mass. Other LPSs of the *E. coli* F583, *E. coli* O111:B4, *S. typhimurium* TV119, and *S. typhimurium* SL 684 strains were purchased from Sigma (St. Louis, MO, USA) and used without further purification.

Labeling of LPSs with Biotin—One milligram of LPS was dispersed by sonication in 1 ml of 100 mM sodium bicarbonate. The dispersion was stirred with 20 μl of *N*-hydroxysuccinimidobiotin in DMSO (50 mg/ml) at room temperature for 4 h. The resultant mixture was dialyzed against TBS (50 mM Tris-HCl buffer containing 100 mM NaCl, pH 7.4). The concentration of the labeled LPS was determined by the phenol-H_2SO_4 coloring method (490 nm) using a calibration curve obtained with the corresponding non-labeled LPS.

Enzyme-Linked Plate Assay for the Binding of HisH with Biotin-Labeled LPSs—A fixed concentration of HisH (0.5 μg/ml) in 100 μl of TBS was adsorbed on 96 flat-bottom wells (Immuno Plate II; Nalge Nunc International, Tokyo) at 4°C for 4 h. After being rinsed with 150 μl of TBS three times, the wells were coated with 0.5% w/v BSA in 150 μl TBS at 4°C for 4 h. The wells were then rinsed three times with 200 μl of wash buffer [TBS containing 0.05% w/v BSA and 0.05% v/v Tween 20] and one time with 200 μl of TBS. Various concentrations of the biotin-labeled LPSs of the *E. coli* C and *S. typhimurium* TV119 (0–10 μg/ml), and *E. coli* F583 and *E. coli* O111:B4 (0–30 μg/ml) strains in 100 μl TBS were added, followed by incubation overnight at 4°C. The wells were rinsed with wash buffer (200 μl) four times, and then streptavidin-peroxidase complex (STP-POD; Zymed, South San Francisco, CA, USA) in 100 μl wash buffer was added, followed by reaction for 3 h at room temperature. The substrate solution (100 μl), comprising *o*-phenylenediamine dihydrochloride (2 mg/ml) and 30% w/v H_2O_2 (1 μl/ml) in 50 mM sodium citrate-phosphate buffer, pH 5.0, was then dropped into the wells at room temperature after removal of excess STP-POD by washing four times with wash buffer (200 μl). Finally, the peroxidase reaction was stopped by the addition of 3 M HCl (50 μl), and then the absorbance at 490 nm was measured, with reference to the absorbance at 630 nm, using an Immuno-Mini NJ-2300 plate reader (Nalge Nunc International).

Fluorometric Titration of HisH with LPSs—Fluorescence spectra of HisH were measured with a Hitachi 650-560 spectrometer. The excitation wavelength λ_{ex} 280 nm, excitation slit width, 1 nm, and emission slit width, 12 nm, were fixed throughout. The fluorescence intensity change caused by the binding of HisH with LPSs was monitored as follows: the LPS solution (typically 0.69 mM) in 50 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.4) was added by means of a microsyringe (5–10 μl) to the solution of HisH (1.5 ml, 0.49 μM) in the same buffer in a quartz cell thermostatted at the desired temperature, and then the fluorescence intensity at the emission wave length, λ_{em}, of 326 nm was recorded after the addition of the LPS solution. The observed fluorescence intensity was corrected for the dilution factor caused by the addition of the LPS solution. The slight fluorescence perturbation by the LPS solution was also corrected for.

RESULTS AND DISCUSSION

Preparation of HisH—The expressed HisH in a cell lysate of *E. coli* M15 (pREP4) harboring the expression vector (Fig. 1, lane 2) was purified as a single band corresponding to the expected molecular weight, 36 kDa, on sodium polyacrylamide-gel electrophoresis (SDS-PAGE) after two step chromatography; Ni-NTA-agarose affinity chromatography (lane 3) and then DEAE-CELLULOFINE A-500

anion-exchange chromatography (lane 4). About 3.6 mg of the purified HisH was obtained from 2 liters of culture. The N-terminal amino acid sequence [MRGSHHHHHHGSDDD] was confirmed.

An attempt to remove the tag sequence from HisH with enterokinase resulted in over-digestion of the formed H protein. However, the N-terminal amino acid sequence of the H protein [MFGAIAGGIA] was confirmed by sequencing of the digested protein material that had been transferred to a membrane from the SDS-PAGE gel. Since the digestion reaction was not very clean, an electrophoretically pure HisH was used in the experiments for detecting the interactions with the LPSs of ϕX174-sensitive and -insensitive strains.

Binding Selectivity of HisH for LPSs in the Enzyme-Linked Plate Assay—E. coli C is one of the native hosts of ϕX174. LPS was extracted from cultivated *E. coli* C cells, its receptor activity toward ϕX174 having been confirmed in the previous study (*16*). The structure of the LPS of *E. coli* C is illustrated on Fig. 2, which has a complete *E. coli* R1 type core oligosaccharide, consisting of two galactopyranosides and three glucopyranosides (*3–5, 18*). The commercially available LPSs which were used in this study are also summarized in Fig. 2. Since some chemical linkages could not be determined unambiguously, the most reasonable structures are presented after reviewing recent structural studies on LPSs (*18–22*). *S. typhimurium* TV119 Ra chemotype was also selected as a ϕX174-sensitive strain, it is having a complete *Salmonella* type core consisting of a pentasaccharide of hexose and a trisaccharide of heptose (*18, 19*). In addition, *E. coli* F583 (Rd_2 chemotype, *20*) and *E. coli* O111:B4 (smooth strain, *21, 22*) were selected as ϕX174-insensitive strains. In order to detect the LPS bound on an assay plate, the above LPSs were labeled with biotin. The free amino group of the phosphoethanolamine moiety would be the linkage point for biotin. The ϕX174 receptor activity of the biotin-labeled LPSs was confirmed by means of the *in vitro* plaque counting assay, *i.e.* the labeled LPSs retained the native receptor activity after labeling (*14*).

A fixed concentration of HisH (0.5 μg/ml) was adsorbed onto a polystyrene assay plate and then subjected to interaction with various concentrations of the biotin-labeled LPSs. The biotin moiety bound to LPS was detected as the orange color (490 nm) of oxidized *o*-phenylenediamine dihydrochloride using the streptavidin-peroxidase complex. As shown in Fig. 3, a dose-dependent increase in ΔAbsorbance at 490 nm was clearly observed for the biotin-labeled LPSs of the ϕX174-sensitive strains, *E. coli* C and *S. typhimurium* TV119, in the concentration range of 0–5 μg/ml. In sharp contrast, the increase in absorbance for those of the ϕX174-insensitive strains, *E. coli* F583 and *E. coli* O111:B4, was low, even when higher concentrations (0–30 μg/ml) were applied. Hence the selective binding of HisH to the

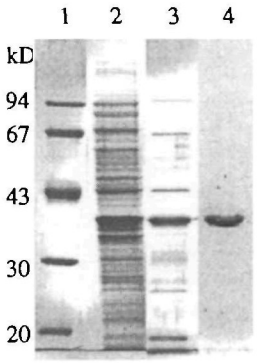

Fig. 1. **Preparation of HisH.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%). Lane 1, M_r markers; lane 2, cell lysate at 3 h cultivation after IPTG induction; lane 3, Ni-NTA fractionated HisH; lane 4, DEAE-CELLULOFINE purified HisH.

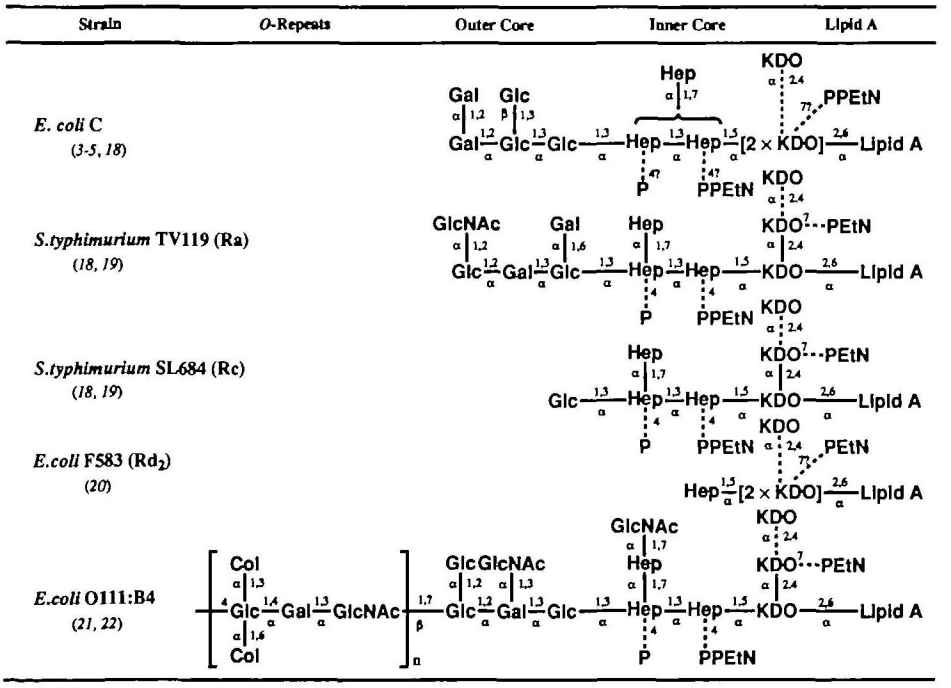

Fig. 2. **Chemical structures of LPSs.** The structures of the LPSs used in the present study are summarized with reference to recent reports (*18–22*). Some chemical linkages could not be determined unambiguously, and dotted lines in the figure indicate non-stoichiometric substitutions.

LPSs of the ϕX174-sensitive strains was observed, this finding being well consistent with the host selectivity on the infection by a whole particle of ϕX174. As a receptor for ϕX174, LPS is required to have the complete sequence of the R-core oligosaccharide and not to have any *O*-repeats (*3*, *5*), and the terminal hexose residue of the pentasaccharide on the outer core is an especially crucial residue for the host selection (*4*). The fact that the LPS of *E. coli* F583 was recognized only weakly is quite reasonable, because its *E. coli* R1-type core lacks all of the hexoses of the outer core and two of the three heptoses of the inner core (*20*). The LPS of *E. coli* O111:B4 also bound weakly to HisH. This LPS has various *O*-repeats in addition to the R-core (*21*, *22*). If the strain lost the *O*-repeats, the corresponding Ra chemotype, having a complete *E. coli* R3-type core, would be ϕX174-sensitive. The LPS of this smooth strain was consequently incompatible with HisH because of the unnecessary *O*-repeats.

In order to determine the affinity of HisH toward the LPSs, the concentrations needed for 50% saturation, $S_{50\%}$ (μg/ml), were calculated by least squaring fitting to Eq. 1, where $[LPS]_0$ is the initial concentration (μg/ml) of the LPS, and ΔAbs_{max} is the maximum change in absorbance that should be observed when all the HisH molecules on the plate form a complex with the LPS.

$$\Delta Abs = \frac{\Delta Abs_{max}[LPS]_0}{S_{50\%} + [LPS]_0} \quad (1)$$

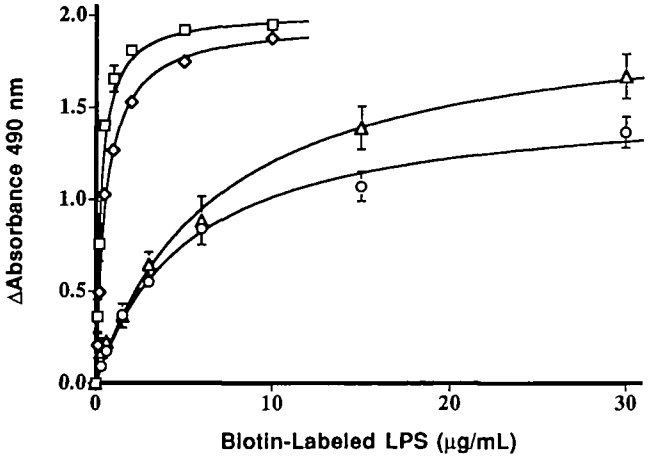

Fig. 3. **Binding of HisH with LPSs of ϕX174-sensitive and -insensitive strains in the enzyme-linked plate assay.** Conditions: A fixed concentration (0.5 μg/ml) of HisH was adsorbed onto an assay plate, followed by treatment with various concentrations of the biotin-labeled LPSs of *E. coli* C (squares), *S. typhimurium* TV119 (Ra) (diamonds), *E. coli* F583 (Rd_2) (circles), and *E. coli* O111:B4 (smooth strain) (triangles) in the 50 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.4) at 4°C overnight. The bound LPSs were detected as the absorbance at 490 nm by means of streptavidine-peroxidase coupled reaction with *o*-phenylenediamine dihydrochloride as a substrate. Standard deviations at the 95% confidence level were calculated for three independent experiments. The $S_{50\%}$ and ΔAbs_{max} values were obtained by least squaring fitting to Eq. 1. The values are for the LPSs of: *E. coli* C ($S_{50\%}$ = 0.290 μg/ml, ΔAbs_{max} = 2.016), *S. typhimurium* TV119 (0.550 μg/ml, 1.966), *E. coli* F583 (5.29 μg/ml, 1.549), and *E. coli* O111: B4 (6.84 μg/ml, 2.029), respectively. The solid lines are theoretical curves drawn according to Eq. 1 using the calculated values.

The solid lines in Fig. 3 are theoretical curves drawn according to Eq. 1 using the calculated values. Because the absolute value of $S_{50\%}$ tended to vary with each assay plate, the relative affinity was calculated by taking the reciprocal numbers of $S_{50\%}$ for the LPSs of *S. typhimurium* TV119 as 53%, *E. coli* F583 as 5.5%, and *E. coli* O111:B4 as 4.2%, respectively, when the affinity of the LPS of *E. coli* C was taken as 100%.

Fluorometric Titration of HisH with LPSs—Fluorometric titration was performed to determine the absolute scale of the affinity of HisH to the LPSs. Contrary to the enzyme-linked plate assay, no labeling of the LPS is needed, because the change in the fluorescence of the protein itself is detected as proof of the binding. Fluorescence spectra of HisH in the absence and presence of the LPS of *E. coli* C were obtained at 22°C (Fig. 4). In the absence of the LPS, the spectrum had an emission peak λ_{max} around 354 nm. On the addition of the LPS solution, the fluorescence intensity around 300–345 nm increased and the peak shifted to the shorter wavelength side. Figure 5 shows the reasonable dependence of the fluorescence intensity increase, ΔF (open circles), at 326 nm on the initial concentration of the LPS, where ΔF is defined as the percentage increase in the fluorescence intensity relative to that in the absence of LPS. An isosbestic point of the fluorescence spectra was seen around 366 nm, which suggests a two-state equilibrium for the binding of LPS and the protein. Thus we built a simple two state model for the bound- and unbound-states of HisH with the LPS as the first step for binding analysis assuming that HisH binds with LPS in a 1:1 stoichiometry and

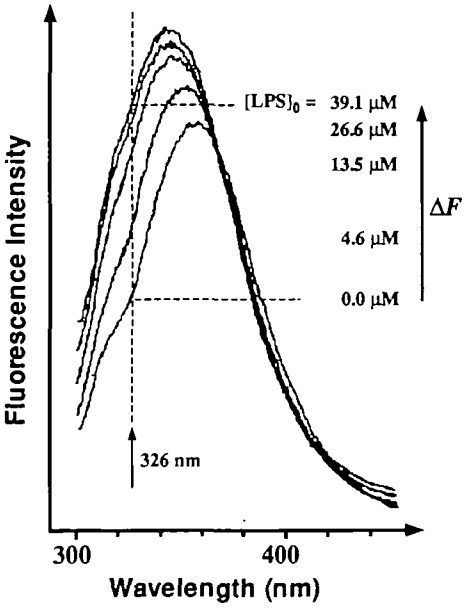

Fig. 4. **Fluorescence spectra of HisH in the absence and presence of the LPS of *E. coli* C.** Conditions: The initial concentration of HisH was 0.49 μM in 1.5 ml of 50 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.4) at 22°C. Spectra were recorded with stepwise additions of the LPS solution (0.69 mM) in the same buffer, the final concentrations of the LPS being 0, 4.6, 13.5, 26.6, and 39.1 μM, respectively. The excitation wavelength λ_{ex} was fixed at 280 nm throughout and the emission wavelength λ_{em} was 300–450 nm. The spectra in the figure were not corrected for the dilution caused by the addition of the LPS solution.

that the initial concentration of LPS is far greater than that of HisH, where $[LPS]_0$ = 0–40 μM and $[HisH]_0$ = 0.49 μM, respectively.[3] The values of the dissociation constant K_d of the complex of HisH with the LPS and the maximum fluorescence intensity increase, ΔF_{max}, which should be observed when all the HisH molecules form a complex, were determined based on Eq. 2.

$$\Delta F = \frac{\Delta F_{max}[LPS]_0}{K_d + [LPS]_0} \quad (2)$$

The values of K_d and ΔF_{max} obtained by least squaring fitting were K_d = 7.02 ± 0.37 μM and ΔF_{max} = 79.8 ± 1.2%, respectively. The solid line in Fig. 5 is the theoretical curve drawn according to Eq. 2 using the above values. The standard Gibbs free energy change ΔG^0 at 22°C was calculated to be –29.1 kJ mol^{-1} from $RT \ln K_d$, where R is the gas constant and T is the absolute temperature. The fluorometric titration of HisH with the LPSs of *S. typhimurium* TV119 (Ra) and *S. typhimurium* SL684 (Rc) was also performed at 25°C and the obtained parameters are summarized in Table I. Less affinity for the LPS of *S. typhimurium* TV119 (K_d = 14.5 μM) compared to that for the LPS of *E. coli* C (K_d = 7.02 μM) was also observed with the enzyme-linked plate assay. Because the affinity of HisH to the LPS of *S. typhimurium* TV119 was calculated to be 49% based on the reciprocal numbers of K_d values, the relative affinity determined with the enzyme-linked plate assay (53%) is consistent with that on fluorometric titration. Although fluorometric titration was also performed for the two φX174-insensitive strains, *E. coli* F583 and *E. coli* O111:B4, the fluorescence changes caused by these two LPSs were so small and noisy that the dissociation constants could not be determined precisely. The K_d value of *S. typhimurium* SL684 (Rc chemotype) was determined to be 49.1 ± 4.8 μM. The relative affinity to HisH was similarly calculated to be 14%, showing a reasonably moderate level of affinity to an R-core oligo-saccharide of the Rc chemotype consisting of one glucose and three heptoses.

In the previous study, we prepared another type of fusion of the H protein with the maltose-binding protein (MBP-H) and analyzed the binding equilibrium between MBP-H and the LPSs of the φX174-sensitive strains (*23*). The dissociation constant of MBP-H with the LPS of *E. coli* C was 21.4 μM, and hence the present affinity of HisH to the same LPS (7.0 μM) is greater than that of MBP-H. Because a small hexa histidine and proteolytic cleavage sequence is fused to the H protein, the steric bulkiness in the case of HisH would be relieved compared to that of MBP-H having a large MBP portion to facilitate the stronger binding to the LPSs.

Driving Force of the Binding—The fluorometric titration of HisH with the LPS of *E. coli* C was also performed at different temperatures, and the K_d values were calculated to be 9.28 ± 0.96 μM (at 15°C) and 5.00 ± 0.32 μM (at 34°C), respectively. The affinity of HisH to the LPS became greater when the temperature was increased. Figure 6 shows the temperature dependence of K_d in a van't Hoff plot, where the slope of the plot shows the standard enthalpy change ΔH^0 is +23.7 kJ mol^{-1}. Based on the standard Gibbs free energy change ΔG^0 of –29.1 kJ mol^{-1} (above mentioned), the standard entropy change ΔS^0 was thus calculated to be 179 J mol^{-1} K^{-1} ($T\Delta S^0$ = 52.8 kJ mol^{-1}) from $\Delta G^0 = \Delta H^0 - T\Delta S^0$. This indicates that the enthalpy part of the standard energy change is unfavorable for binding, consequently a very large entropy change should compensate for the enthalpy. It was thereby shown that the binding of HisH with the LPS is an entropy-driven reaction. When we take the inevitable decrease in the translational and rotational entropy caused by the binding of two molecules to form one molecule into account, the entropy-driven-type character of the binding of LPS with HisH is more evident.

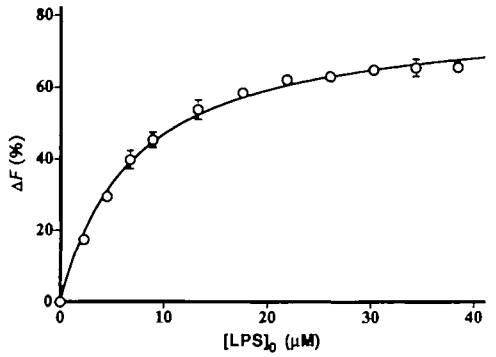

Fig. 5. **Fluorometric titration of HisH with the LPS of *E. coli* C.** The relative fluorescence intensity increase at 326 nm (ΔF%) was measured and plotted against the LPS concentrations with correction for the dilution factor. Slight perturbation of the baseline caused by LPS was also corrected. Standard deviations at the 95% confidence level were calculated for more than three independent experiments. The K_d and ΔF_{max} values were obtained by least squaring fitting to Eq. 2. The solid line is the theoretical curve drawn according to Eq. 2, using the values of K_d = 7.02 μM and ΔF_{max} = 79.84%.

TABLE I. **Parameters determined on fluorometric titration of HisH with LPSs.**

Strain	Temp (°C)	K_d (μM)	ΔF_{max} (%)
E. coli C	15	9.28 ± 0.96	94.8 ± 3.4
E. coli C	22	7.02 ± 0.37	79.8 ± 1.2
E. coli C	34	5.00 ± 0.32	64.5 ± 1.0
S. typhimurium TV119 (Ra)	25	14.46 ± 1.26	95.2 ± 3.4
S. typhimurium SL684 (Rc)	25	49.06 ± 4.76	80.0 ± 5.1

Figure after ± is standard deviation. The molecular weights of the LPSs were calculated based on the chemical structures in Fig. 2 for *E. coli* C (4324), *S. typhimurium* TV119 Ra (4525), and *S. typhimurium* SL684 Rc (3834), respectively.

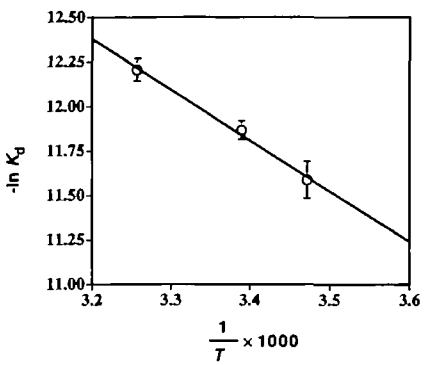

Fig. 6. **Temperature dependence of the dissociation constant K_d for HisH and the LPS of *E. coli* C.**

The molecular binding affinity B, which is the unitary part of the standard affinity $-\Delta G^0$, was estimated to be *ca.* 39 kJ mol^{-1} at 22°C.

The fluorescence spectrum of HisH had a peak λ_{max} around 354 nm in the absence of LPS. This indicates that some or all the three tryptophan residues in HisH (W131, W309, and W313) are exposed to the hydrophilic solvent water, because the λ_{max} in such a high field is similar to that of an aqueous solution of free tryptophan. In the presence of the LPS of *E. coli* C, a large shift of λ_{max} to the shorter wavelength side occurred along with a large increase in the fluorescence intensity. This is attributable to the isolation of the tryptophan residues from the solvent water in a hydrophobic environment caused by the interaction with the LPS. Moreover, such an increase in the fluorescence intensity and a shift of the emission peak were also observed when the excitation wavelength was changed from 280 to 295 nm in order to prevent the overlapped excitation of tyrosine residues, and thus the observed fluorescence response accordingly represented the behavior of tryptophan residues.

We are seeking the reason for the observed quite large entropy change on the interaction of HisH with the hydrophobic part of LPS.[4] The isolation of the exposed hydrophobic region of HisH from the solvent water through binding with LPS would result in relaxation of the water molecules, which are restricted to the exposed hydrophobic surface, and thus an increase in total entropy. Consequently, this large entropy increase is the main driving force of the binding. The spike H protein was reported to penetrate through the membrane of a host cell along with the phage DNA as a piloting protein (*12*) and to promote replication of the phage DNA (*24*). The surface of the H protein would accordingly be hydrophobic in order to intermingle with the lipophilic outer membrane of the host cell. The specific recognition of receptor LPSs by the spike H protein, proved in this study, is quite reasonable as to the functioning of H as a piloting protein, which would be driven by the hydrophobic interaction with LPS as a major constituent of the outer membrane of host cells.

Lindberg *et al.* (*25*) reported that the association constant K_a for a whole particle of bacteriophage G13 with the oligosaccharide part of the LPS of *E. coli* C, which was prepared by weak acid hydrolysis of the LPS, was 1.3×10^7 M^{-1} (*i.e.* $K_d = 7.7 \times 10^{-8}$ M). A whole G13 particle showed stronger affinity to the oligosaccharide part of the LPS than that of the H protein of ϕX174 to the LPS ($K_d = 7.0 \times 10^{-6}$ M), even when the LPS had lost the lipophilic lipid A part. Although G13 belongs to the same microvirus family as ϕX174, its host range is wider than that of ϕX174 (*5*). The difference in affinity would correspond to the difference between the isolated H protein itself and a whole particle with the co-operation of H with other capsid proteins. Recent X-ray crystallography of ϕX174 particles revealed that the spike structure was formed by a pentamer of the G protein, and a part of the uninterpreted H protein was thought to be inside the cavity (*13*). Together with our experimental finding that the engineered G protein (HisG) also bound with the receptor LPSs, the G protein could also participate in the recognition of LPS. Thus, further characterization of the binding of the spike H as well as the G protein is in progress to understand the mechanism of host-recognition by this simple and fundamental virus.

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REFERENCES

1. Hayashi, M., Aoyama, A., Richardson, D.L., Jr., and Hayashi, M.N. (1988) Biology of the bacteriophage ϕX174 in *The Bacteriophages* (Calendar, R., ed.) Vol. 2, pp. 1–71, Plenum Press, New York and London
2. Incardona, N.L. and Selvidge, L. (1973) Mechanism of adsorption and eclipse of bacteriophage ϕX174 II. Attachment and eclipse with isolated *Escherichia coli* cell wall lipopolysaccharide. *J. Virol.* **11**, 775–782
3. Jazwinski, S.M., Lindberg, A.A., and Kornberg, A. (1975) The lipopolysaccharide receptor for bacteriophages ϕX174 and S13. *Virology* **66**, 268–282
4. Feige, U. and Stirm, S. (1976) On the structure of the *Escherichia coli* C cell wall lipopolysaccharide core and on its ϕX174 receptor region. *Biochem. Biophys. Res. Commun.* **71**, 566–573
5. Lindberg, A.A. (1977) Bacterial surface carbohydrates and bacteriophage adsorption in *Surface Carbohydrates of the Prokaryotic Cell* (Sutherland, I., ed.) Chap. 8, pp. 289–356, Academic Press, London
6. Brown, D.T., MacKenzie, J.M., and Bayer, M.E. (1971) Mode of host cell penetration by bacteriophage ϕX174. *J. Virol.* **7**, 836–846
7. Mano, Y., Kawabe, T., Komano, T., and Yazaki, K. (1982) Involvement of a cell envelope component of *Escherichia coli* in the early stages of infection with bacteriophage ϕX174. *Agric. Biol. Chem.* **46**, 2041–2049
8. Edgell, M.H., Hutchinson III, C.A., and Sinsheimer, R.L. (1969) The process of infection with bacteriophage ϕX174 XXVIII. Removal of the spike proteins from the phage capsid. *J. Mol. Biol.* **42**, 547–557
9. Sinsheimer, R.L. (1968) Bacteriophage ϕX174 and related viruses. *Progr. Nucleic Acid Res. Mol. Biol.* **8**, 115–169
10. Siegel, J.E.D. and Hayashi, M. (1969) ϕX-174 bacteriophage structural mutants which affect deoxyribonucleic acid synthesis. *J. Virol.* **4**, 400–407
11. Newbold, J.E. and Sinsheimer, R.L. (1970) Process of infection with bacteriophage ϕX174 XXXIV. Kinetics of the attachment and eclipse steps of the infection. *J. Virol.* **5**, 427–431
12. Jazwinski, S.M., Lindberg, A.A., and Kornberg, A. (1975) The gene H spike protein of bacteriophages ϕX174 and S13 I. Functions in phage-receptor recognition and in transfection. *Virology* **66**, 283–293
13. McKenna, R., Xia, D., Willingmann, P., Ilag, L.L., Krishnaswamy, S., Rossmann, M.G., Olson, N.H., Baker, T.S., and Incardona, N.L. (1992) Atomic structure of single-stranded DNA bacteriophage ϕX174 and its functional implications. *Nature* **355**, 137–143
14. Suzuki, R., Inagaki, M., Karita, S., Kawaura, T., Kato, M., Nishikawa, S., Kashimura, N., and Morita, J. (1999) Specific interaction of fused H protein of bacteriophage ϕX174 with receptor lipopolysaccharides. *Virus Res.* **60**, 95–99
15. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY
16. Inagaki, M., Kato, M., Ohsumi, Y., Kaitani, K., Nishikawa, S., and Kashimura, N. (1995) Simple preparation of large amount of lipopolysaccharide with receptor activity for bacteriophage ϕX174 from *Escherichia coli* C strain. *Bull. Fac. Bioresour. Mie Univ.* **15**, 33–40
17. Galanos, C., Lüderits, O., and Westphal, O. (1969) A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* **9**, 245–249
18. Holst, O. and Brade, H. (1992) Chemical structure of the core region of lipopolysaccharides in *Bacterial Endotoxic Lipopolysaccharides* (Morrison, D.C. and Ryan, J.L., eds.) Vol. 1: Molecular Biochemistry and Cellular Biology, pp. 135–170, CRC Press,

Boca Raton
19. Nnalue, N.A., Lind, S.M., and Lindberg, A.A. (1992) The disaccharide L-α-D-heptose1→7-L-α-D-heptose1→ of the inner core domain of *Salmonella* lipopolysaccharide is accessible to antibody and is the epitope of a broadly reactive monoclonal antibody. *J. Immunol.* **149**, 2722–2728
20. Schmidt, G., Jann, B., and Jann, K. (1970) Immunochemistry of R lipopolysaccharides of *Escherichia coli*. Studies on R mutants with an incomplete core, derived from *E. coli* O8: K27. *Eur. J. Biochem.* **16**, 382–392
21. Jann, K. and Jann, B. (1984) Structure and biosynthesis of *O*-antigens in *Handbook of Endotoxin* (Rietschel, E.T., ed.) Vol. 1: Chemistry of Endotoxin, pp. 138–186, Elsevier Science Publishers, Amsterdam
22. Haishima, Y., Holst, O., and Brade, H. (1992) Structural investigation on the lipopolysaccharide of *Escherichia coli* rough mutant F653 representing the R3 core type. *Eur. J. Biochem.* **203**, 127–134
23. Inagaki, M., Maeyama, K., Handa, H., Tanaka, A., Karita, S., Nishikawa, S., and Kashimura, N. (2000) Evaluation of specific binding of H protein of bacteriophage ϕX174 fused with maltose-binding protein toward receptor lipopolysaccharides. *J. Biochem. Mol. Biol. Biophys.* **4**, 59–64
24. Jazwinski, S.M., Marco, R., and Kornberg, A. (1975) The gene H spike protein of bacteriophages ϕX174 and S13 II. Relation to synthesis of the parental replicative form. *Virology* **66**, 294–305
25. Bruse, G.W., Wollin, R., and Lindberg, A.A. (1989) Interaction between phage G13 and its oligosaccharide receptor studied by equilibrium dialysis. *J. Mol. Recognit.* **2**, 18–24