ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 3, pp. 332-339. © Pleiades Publishing, Inc., 2006.
Original Russian Text © V. N. Davidova, G. A. Naberezhnykh, I. M. Yermak, V. I. Gorbach, T. F. Solov'eva, 2006, published in Biokhimiya, 2006, Vol. 71, No. 3, pp. 417-425.

Determination of Binding Constants of Lipopolysaccharides of Different Structure with Chitosan

V. N. Davidova*, G. A. Naberezhnykh, I. M. Yermak, V. I. Gorbach, and T. F. Solov'eva

Pacific Institute of Bioorganic Chemistry, Far-Eastern Division of the Russian Academy of Sciences, pr. 100-letiya Vladivostoka 159, 690022 Vladivostok, Russia; fax: (4232) 314-050; E-mail: viktoria@piboc.dvo.ru

Received April 20, 2005

Abstract—The interaction of endotoxins—lipopolysaccharides (LPS) different in degree of the O-specific chain polymerization—with 20- and 130-kD chitosan was studied using the competitive binding of LPS with the complex of chitosan–anionic dye (tropaeolin 000-2) and the direct binding of ^{125}I-labeled LPS with chitosan immobilized on Sepharose 4B. The interaction of 20-kD chitosan with LPS was non-cooperative, and immobilization of the polycation on Sepharose resulted in its binding to ^{125}I-labeled LPS with a positive cooperativity. The interaction of LPS possessing a long O-specific chain with 130-kD chitosan was characterized by negative cooperativity. Binding constants of LPS with the polycation and the number of binding sites per amino group of chitosan were determined. The interaction affinity and stoichiometry of the LPS–chitosan complexes significantly depend on the LPS structure and concentration in the reaction mixture. The increase in the length of carbohydrate chains of LPS results in increase in the binding constants and decrease in the bound endotoxin amount.

DOI: 10.1134/S0006297906030151

Key words: lipopolysaccharide, chitosan, tropaeolin 000-2, binding constants, O-specific chain of LPS

Lipopolysaccharide (LPS) endotoxins are important factors of pathogenicity of gram-negative bacteria. The ability of endotoxins to interact with molecules of a macroorganism, in particular, with soluble serum proteins, serum lipoproteins, and various cellular receptors determine many biological features of endotoxins [1]. Moreover, on entrance of the bacterial cell into the body, LPS become a target for polycationic antibacterial agents, such as bactericidal proteins of the host and antibiotics used to treat the infection [2].

The molecular mechanism of the interaction of LPS with polycations is still insufficiently clear. Interactions between negatively charged groups of the endotoxin and positive groups on the polymers that bind to it are generally thought to underlie this mechanism [2, 3]. However, there are also data indicating that formation of stable endotoxin–polycation complexes is a result of hydrophobic interactions [4]. Despite great interest in this problem, there are only scarce data on the stoichiometry of the complexes and on the effect of the endotoxin structure on the complexing [3, 5, 6]. This is mainly due to the nature of LPS molecules themselves, which consist of hydrophobic (lipid A) and hydrophilic (O-specific polysaccharide and core oligosaccharide) regions. The lipid and the inner part of the endotoxin core contain negatively charged groups (phosphate, pyrophosphate, carboxyl [7]) that are mainly responsible for their interaction with various polycations.

Due to amphiphilicity, LPS in solution can produce aggregations with supramolecular organization depending on both conditions of dissolving (temperature, polymer concentration) and the endotoxin structure [1, 6]. This in turn influences the package density of macromolecules in the LPS aggregations and availability of charged particles for interaction with polycations [8].

We have earlier shown that LPS interact with the natural polycation chitosan and produce stable complexes of different stoichiometry. The composition of these complexes and their formation depend on both the LPS structure and parameters of the medium where the complexing occurs [9, 10].

Abbreviations: LPS) lipopolysaccharides; Ch-HM) high molecular weight chitosan (130 kD); Ch-LM) low molecular weight chitosan (20 kD); D_0 and D_{exp}) absorption of solution before and after addition of LPS; ΔD_{max}) absorption difference of the tropaeolin–chitosan complex and tropaeolin–chitosan–LPS solution corresponding to chitosan saturation with LPS; Q) saturation degree of chitosan with LPS molecules; K_b) binding constant.

* To whom correspondence should be addressed.

The present work is a continuation of studies in this line. Its purpose was to study the interaction of endotoxins possessing differently polymerized O-specific chain with chitosan in diluted solutions and determine the binding constants of LPS with the polycation using approaches of the endotoxin competitive binding with the complex of chitosan–anionic dye (tropaeolin 000-2) and also the direct binding of ^{125}I-labeled LPS with chitosan immobilized on Sepharose.

MATERIALS AND METHODS

Chloramine T (Serva, Germany), Sepharose 4B (Pharmacia, Sweden), Na^{125}I (Izotop, Russia), and the dye tropaeolin 000-2 (Orange-2) (Soyuzreactiv, Russia) were used. The dye was purified by recrystallization from ethanol. All other reagents were of chemical purity (Reakhim, Russia) and were used without additional purification.

Isolation and purification of LPS. LPS were isolated from the bacterium *Yersinia pseudotuberculosis* 1B serovar. LPS-1 was extracted from the microorganisms grown at 4°C with a mixture of phenol–chloroform–petroleum ether as described in [11], and LPS-2 was obtained by extraction with 45% hot phenol as described in [12]. R-Forms of LPS were extracted from the bacterium cultured at 37°C with hot aqueous phenol as described in [12]. LPS-3 was prepared as described in [13]. The fraction precipitated with acetone from the phenol layer was used in experiments. The resulting LPS were purified from nucleic acids by ultracentrifugation at 105,000*g*. The monosaccharide composition and polymerization degree of the O-specific chain of LPS were established by GLC of monosaccharides prepared as polyol acetates according to [14], with xylose as an internal standard.

Radioactive labeling of LPS. LPS-3 (2 mg) was dissolved in 0.2 ml of water, supplemented with 4 mg bromocyanogen, and the mixture was stirred for 8 min at 0°C; the pH value of 10 was maintained by addition of 1 N NaOH. Then 1 mg tyramine hydrochloride was added, the pH was adjusted to 8.0 with 0.2 M $NaHCO_3$, and the mixture was stirred for 16 h at 20°C. The tyraminated LPS was isolated by gel chromatography on Sephadex G-50 in phosphate buffer (pH 5.0). The tyramination determined by absorption at 280 nm was 2 mol tyramine per 1 mol LPS. Tyraminated LPS was iodinated with chloramine T as described in [15]. The specific radioactivity of ^{125}I-labeled LPS was $2 \cdot 10^4$ cpm/μg.

Preparation of chitosans. Chitosans with molecular weights of 130 and 20 kD (Ch-HM and Ch-LM, respectively) were prepared as described earlier [16].

Titration of the tropaeolin 000-2–chitosan complex with LPS solution. To 80 μl of 0.005 M phosphate buffer (pH 5.0), 40 μl of tropaeolin solution (0.08 mg/ml) and 20 μl of chitosan solution (100 μg/ml) were added. The mixture was incubated for 20 min at 20°C. Then the resulting complex was titrated with LPS solution (200 μg/ml) preincubated at 37°C for 48 h, by successively adding portions of 5-10 μl. The mixture was incubated at 37°C for 2 h (LPS-2, LPS-3) or 18 h (R-LPS, LPS-1). The absorption was determined with a spectrophotometer μQuant Bio-TEK Instruments (USA) at 483 nm in three parallel samples, and the mean arithmetic value was calculated. The value of $\Delta D = D_{exp} - D_0$ was calculated where D_0 and D_{exp} were absorptions of the solutions before and after addition of LPS, respectively. The values of ΔD_{max} and K_b were determined from the Scatchard dependence in $\Delta D/C_{LPS}$ versus ΔD coordinates. The chitosan saturation (Q) with LPS molecules was determined from the ratio $\Delta D/\Delta D_{max}$.

The degree of cooperativity (h) was determined from the Hill equation in logarithmic form [17]:

$$\log(Q/1 - Q) = h \log[C_{LPS}] - \log[K_b].$$

The number of binding sites on the endotoxin molecule per amino group of chitosan was assessed from the saturation curve plotting a tangent to the point that corresponded to the maximal change in the reaction mixture absorption. Then the LPS concentration corresponding to chitosan saturation with the endotoxin was determined.

Preparation of Ch-LM-Sepharose. To 4 ml of water-washed Sepharose 4B 2 ml of $NaIO_4$ solution (15 mg/ml) was added, and the mixture was stirred for 60 min and then supplemented with 0.1 ml of ethylene glycol. After 10 min, the Sepharose was washed in water and supplemented with solution of Ch-LM (20 mg in 1 ml of 0.001 M sodium carbonate buffer containing 0.9% NaCl (pH 8.0)). The mixture was stirred for 24 h, supplemented with 60 mg $NaBH_4$, maintained for 1 h, and washed with water on a filter. To determine the amount of chitosan bound to Sepharose, 1 ml of the sorbent was dried and hydrolyzed with 2 ml of concentrated HCl in a sealed ampule at 100°C for 4 h. The hydrolyzate was filtered and evaporated. The amount of free glucosamine was determined by the Elson–Morgan method [18]. The amount of glucosamine corresponding to the amount of immobilized chitosan was 1.2 mg per ml of the sorbent.

Determination of equilibrium binding constants of ^{125}I-labeled LPS-3 with immobilized chitosan. Ch-LM-Sepharose was washed in 0.002 M phosphate buffer (pH 5.0) and suspended in an equal volume of this buffer. To 0.05 ml of Ch-LM-Sepharose (the chitosan concentration in the reaction mixture was 0.002 M), varied volumes of 0.001 M solution of ^{125}I-labeled LPS were added (the concentration was varied from 0.3 to 7 mM). The medium volume was adjusted to 0.4 ml with phosphate buffer, and the mixture was shaken for 4 h at 37°C and then centrifuged. The precipitated sorbent was washed. Then the radioactivity of the bound LPS was determined using a RIA-GAMMA gamma-counter (LKB, Sweden).

To determine the nonspecific sorption of ^{125}I-labeled LPS on Ch-LM-Sepharose, the binding was performed in the presence of a 100-fold excess of unlabeled homologous LPS (0.03-0.7 M). The nonspecific binding was 9-12%.

The binding data were plotted in Scatchard coordinates, and the analytical plot suggested positive cooperativity of the ^{125}I-labeled LPS interaction with immobilized Ch-LM. The binding parameters were calculated with a computer program based on approaches proposed in [19] using the following equation:

$$r/[S] = aK_b^h[S]^{h-1}/(1 + K_b^h[S]^h),$$

where r is the ratio of the bound LPS concentration to the concentration of added chitosan, S is the concentration of free LPS, a is the number of binding sites on a chitosan molecule, K_b is the binding constant, and h is the Hill coefficient.

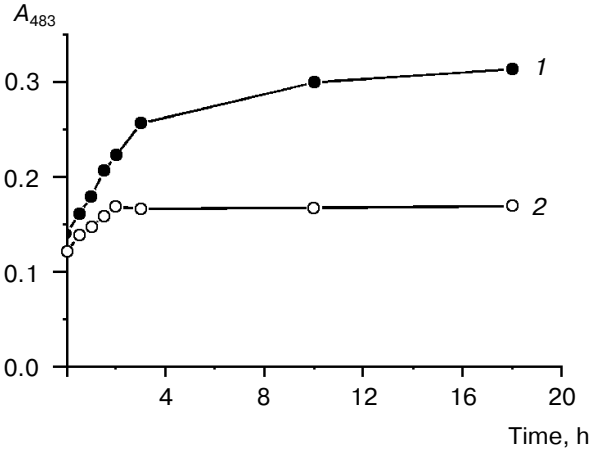

Fig. 1. Dependence of the absorption of LPS–Ch-LM–tropaeolin mixture on the time of incubation at 37°C at the point of chitosan saturation: *1*) R-LPS; *2*) LPS-2. To 100 µl of the Ch-LM–tropaeolin complex the necessary amount of LPS solution (200 µg/ml) was added, the resulting mixture was incubated at 37°C, and the value of A_{483} was determined every hour.

RESULTS

Characteristics of initial components of the complex. LPS isolated from *Y. pseudotuberculosis* and different in the O-specific chain polymerization degree were used. LPS with 2.4, 13, and 24 degrees of the O-specific polysaccharide polymerization are denoted, respectively, as LPS-1, LPS-2, and LPS-3. In addition, LPS without O-specific chains (R-LPS) was also used in this work. The polymerization degree of the repeated links of the O-specific polysaccharide and molecular weights of the corresponding LPS are presented in Table 1. To perform radioligand analysis, LPS-3 was radiolabeled with ^{125}I. Parameters of the complexing were calculated based on molecular weights of LPS molecules.

Two samples of chitosan were used, with 4% acetylation degree of glucosamine residues and molecular weights of 20 and 130 kD. By analytical centrifugation, the chitosan samples were shown to have a rather narrow distribution of molecular weights [16].

Because of chemical nature of the components, LPS and chitosan interact with involvement of charged groups (amino groups of chitosan, on one hand, and carboxyl and phosphate groups of LPS, on the other hand). As a result, the pH value of the medium, which determines the ionization degree of these groups, could significantly influence the binding of the components. In fact, we found that the LPS–chitosan complex was produced at the pH range of 4.0-7.0. Therefore, to study the binding, the pH value of 5.0 was chosen.

Table 1. Characteristics of LPS used in this work

Sample of LPS	*n**	Molecular weight, kD
R-LPS	—	4.3
LPS-1	2.4	6.2
LPS-2	13	12.5
LPS-3	24	23.5

* Polymerization degree of O-specific chain.

Interaction of chitosan–tropaeolin complex with LPS and determination of polymer binding constants. Binding constants of the endotoxin with the polycations were determined using the lipopolysaccharide replacement of the anionic dye in its complex with chitosan and recording the released dye.

The interaction of four LPS samples (R-LPS, LPS-1, LPS-2, and LPS-3) with Ch-LM and Ch-HM was studied using the anionic dye tropaeolin 000-2 (sodium salt of 4-(2-hydroxy-1-naphthyl)benzenesulfonic acid) in 0.005 M phosphate buffer (pH 5.0). As shown previously [20], the dye was quantitatively bound to every ionized amino group in the polycation molecule, and this significantly decreased the absorption (to 70%) in the absorption maximum of the dye at 483 nm. On addition of LPS solution to the tropaeolin–chitosan complex, the absorption of the reaction mixture increased to the value corresponding to that of the free dye. This indicated the lipopolysaccharide replacement of the dye in its complex with chitosan and the endotoxin binding with the polycation. No effect of LPS on the absorption of tropaeolin was recorded.

It should be noted that the endotoxin changed the optical absorption of the tropaeolin–polycation solution only if free LPS had been partially pre-dissociated at

37°C for 48 h, whereas at 25°C the highly aggregated endotoxin failed to displace the dye from its complex with chitosan. To reach the constant value of the optical absorption of the solution on titration of the tropaeolin–chitosan–LPS complex, a certain time was required. At 37°C, the absorption value of the reaction mixture became constant after 1.5-2 h when LPS with the O-specific polysaccharide chain (LPS-1, LPS-2, and LPS-3) were used and only after 18 h in the case of R-LPS (Fig. 1).

Data on the LPS binding with Ch-LM presented as linear Scatchard plots (Fig. 2a) suggest an interaction with a certain type of binding sites and the absence of cooperativity during the interaction. Values of the cooperativity coefficients determined from the Hill plot are close to unity, confirming our hypothesis (Table 2).

In the case of complexes with Ch-HM, the Scatchard plots were linear only for the polycation complexes with R-LPS and LPS-1 (Fig. 2b). For the LPS with the long O-specific chain (LPS-2 and LPS-3) the Scatchard plot (Fig. 2c) had a characteristic inflection point that could be caused by both the presence of two type binding sites (with high and low affinity) and negative cooperativity.

On assuming the independence of the binding sites on chitosan, the chitosan binding constants with LPS were determined from the Scatchard and Hill plots. Their values are presented in Table 2.

The LPS concentration corresponding to the point of chitosan saturation with endotoxin was determined from the saturation curves, and on this basis the number of binding sites at the saturation point was calculated (Table 2).

Radioligand determination of the LPS binding constants with chitosan. Radioligand analysis with ^{125}I-labeled LPS-3 was also used to study the interaction of LPS with Ch-LM. To separate the chitosan-bound LPS from the unbound LPS, Ch-LM was immobilized on Sepharose 4B. Chitosan was covalently bound to Sepharose by activation of Sepharose with bromocyanogen and divinylsulfone and by oxidation with periodate. The latter approach was the best, and it allowed us to determine the polycation binding at weakly alkaline pH.

To determine K_b and the binding sites for LPS on chitosan, an equal amount of immobilized chitosan was titrated with the radioactive ligand ^{125}I-labeled LPS-3. Both the binding and titration of the tropaeolin–chitosan–LPS complex were performed at 37°C, and the reaction mixtures were incubated for 4 h to achieve equilibrium.

The Scatchard plot describing the interaction of Ch-LM with ^{125}I-labeled LPS-3 (Fig. 3) has an ascending branch in the region of low concentrations of the bound ligand, and this suggests positive cooperativity in the immobilized chitosan binding with LPS-3. In fact, in this case the cooperativity coefficient (the Hill coefficient) is higher than unity ($h = 1.8$).

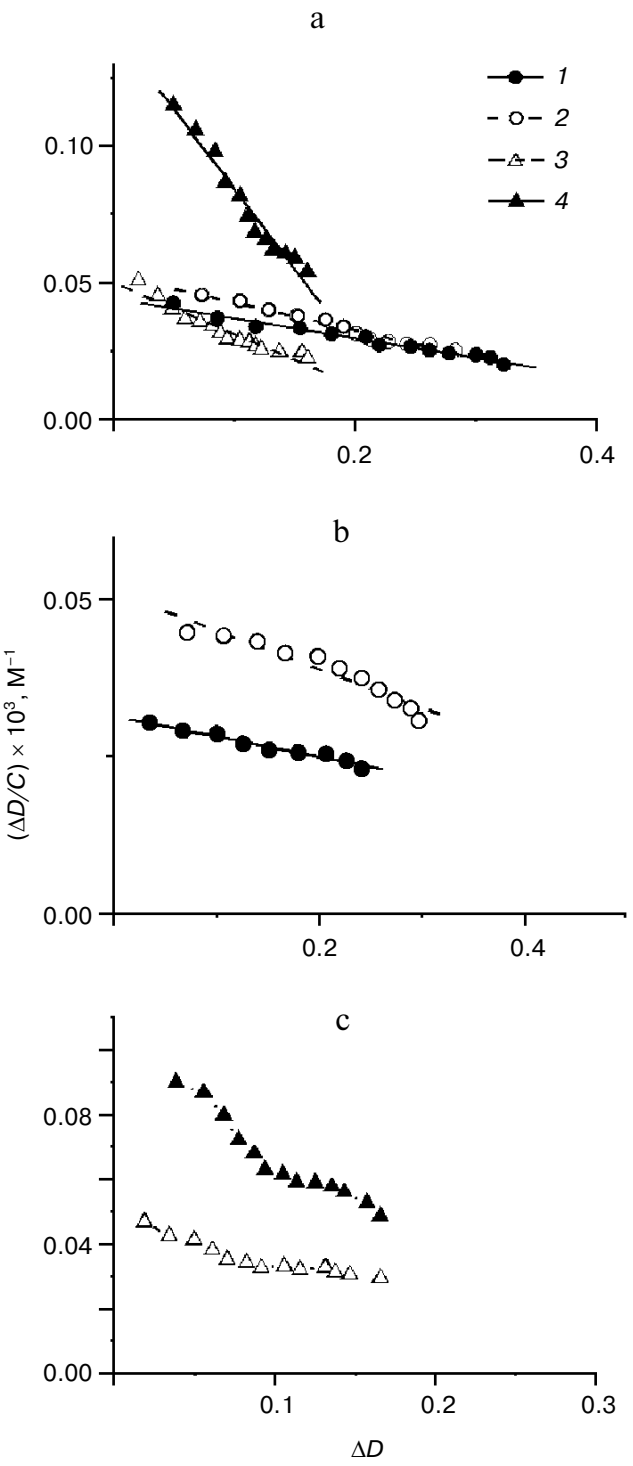

Fig. 2. Scatchard plots for the binding of LPS with Ch-LM (a) and Ch-HM (b and c): *1*) R-LPS; *2*) LPS-1; *3*) LPS-2; *4*) LPS-3. The chitosan–tropaeolin complex (100 μl) was titrated with LPS solution (200 μg/ml), on successive addition of 5-10 μl portions. The absorption was determined at 483 nm in three parallel samples, and the arithmetic mean was calculated. *C* is concentration of the added LPS, ΔD is the difference between the optical absorption of the chitosan–tropaeolin solution before and after the addition of LPS.

Table 2. Parameters of the LPS binding with Ch-HM and Ch-LM

Complex	$K_b \times 10^5$, M (Scatchard)	$K_b \times 10^5$, M (Hill)	$K_b \times 10^5$, M (mean value)	*h*	Number of LPS moles per NH_2-group of chitosan
Ch-LM					
R-LPS	0.759	0.791	0.775	1.020	0.26
LPS-1	1.015	1.009	1.012	1.000	0.19
LPS-2	1.840	1.753	1.797	0.9748	0.11
LPS-3	5.930	6.015	5.973	1.016	0.04
[^{125}I]-LPS-3	3.5	—	—	1.8	0.03
Ch-HM					
R-LPS	0.331	0.326	0.329	1.000	0.17
LPS-1	0.617	0.594	0.606	0.997	0.13
LPS-2	—	0.504	—	0.929	0.09
LPS-3	—	2.820	—	0.974	0.05

The binding constants and number of binding sites on chitosan were calculated on the basis of the cooperative interaction model (Table 2). For LPS-3 the K_b value was $3.5 \cdot 10^5$ M^{-1}. Note that notwithstanding some differences in the K_b values obtained for different LPS, they are on average rather similar.

DISCUSSION

We have earlier shown [10] that LPS and chitosan interact with production of soluble complexes. In the present work the effect of the length of the O-specific polysaccharide endotoxin on its interaction with the polycation and their binding constants were determined. We used LPS isolated from the same strain of the bacterium *Y. pseudotuberculosis*, and this allowed us to obtain a number of samples with the same chemical structure, which were different only in the polymerization degree of the O-chains.

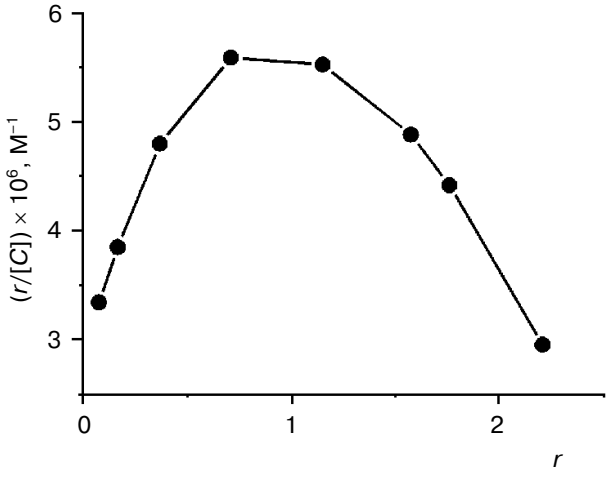

Fig. 3. Scatchard plot of the binding of Ch-LM with ^{125}I-labeled LPS-3. To chitosan bound with 0.05 ml of Sepharose 4B, increasing concentrations of LPS (0.3-0.75 μM) in 0.002 M sodium phosphate buffer (pH 5.0) were added. [*C*] is concentration of free LPS, *r* is ratio of bound LPS concentrations to chitosan.

We found that the LPS were bound on the charged amino groups of chitosan. This was concluded because the endotoxin displaced negatively charged molecules of the dye tropaeolin from its complex with the polycation and the interaction occurred only at pH values from 4 to 7 where amino groups of chitosan were ionized. The interaction was specific, because the binding sites on chitosan were saturated on gradual addition of the ligand, which was demonstrated by the saturation curves (data not presented). Moreover, it was shown by the radioligand analysis that unlabeled LPS considerably inhibited the binding of radioactive LPS with the polycation.

The presented findings suggested that the interaction under consideration is rather complicated. This was manifested even at the initial stage of the process, which required LPS aggregations to be pre-dissociated at elevated temperature for 24 h. The further binding could also be realized only at 37°C, and more hydrophobic endotoxins, short-chained or free of O-chains, had to be incubated in the mixture for a significantly longer time (18 h) than LPS with the long O-chains (Fig. 1). This seems to be associated with the dissociation kinetics of LPS aggregations. Shortening of the O-specific chain is known to be accompanied by an increase in the rigidity or package density of the inner region of the polar head (KDO-phosphate-glucosaminebiose) [21]. Moreover, chitosan dis-

played higher affinity for more hydrophilic LPS-2 and LPS-3. This was indicated by values of the dissociation constants (Table 2) (an increase in the length of the O-specific chain of LPS was associated with increase in its affinity for chitosan). Thus, LPS-3 with the longest O-specific chain had the highest binding constant to the polycation.

The order of the binding constants (10^5 M) is in consistence with values of these parameters found by other authors for LPS complexes with other polycations (human serum albumin [22] and polymyxin [2]).

The complexing depended not only on the LPS structure but also on the molecular weight of the chitosan. Thus, on the interaction of LPS-2 and LPS-3 with Ch-HM (Fig. 2c) the Scatchard plot deviates from a straight line.

It is difficult to interpret curvilinear Scatchard plots in the framework of an adequate model of interaction in the case of such a complicated system as LPS–chitosan, where LPS is a macromolecular, potentially polyvalent ligand with a slight heterogeneity and ability for aggregation in aqueous medium.

The Scatchard plot appearance as a concave curve can indicate the presence of two independent binding sites on the acceptor (chitosan), multivalency of the ligand (LPS), or the binding of components with negative cooperativity [23]. The Hill coefficient value in our case (Table 2) also fails to give an unambiguous answer to this question. The $h < 1$ value can indicate the negative cooperativity or the existence of two types of binding sites with similar values of the interaction constants [17].

The existence of two independent binding sites on chitosan is unlikely because the polycation contains only amino groups with predominantly the same environment. The concave shape of the Scatchard curves can be also due to polyvalency of the ligand. It is known that LPS from the pseudotuberculosis bacteria contain a number of nonequivalent negatively charged groups, which can bind to the polycation [24]. The two binding constants are likely to correspond to these interactions. All LPS used in the experiments had the same number and nature of such groups, thus the observed differences in the shape of the curves cannot be explained in the framework of the above-described model. A possible shielding of the charged groups with polysaccharide O-chains, which could cause the difference in the binding properties of different LPS, also complicates the interpretation of the findings.

Our results of computer-aided modeling of the LPS–chitosan interaction [25] have shown that the binding with only one phosphate in the first position of glucosamine is most likely, whereas the interaction on the other charged particles is energetically unfavorable. Similar data, in particular, on the involvement of only one LPS phosphate also in the first position of LPS glucosamine during its interaction with a pulmonary protein were obtained by Augusto et al. [26]. These authors have shown the importance of the lipid molecule conformation during the complexing. The protein recognizes only a specific conformation of the lipid molecule, and the distance between phosphorus and nitrogen atoms is very significant. This is consistent with our data on the effect of temperature on the complexing. At 37°C, the supramolecular organization of the endotoxin was rearranged because of melting of acyl chains [27], and the endotoxin molecule seemed to acquire a conformation advantageous for interaction with the polycation.

Thus, we suggest that the concave shape of the Scatchard curves of LPS-2 and LPS-3 binding with Ch-HM can be a result of the mutual repulsion of ligands, or anti-cooperative interaction. Large ligands sterically prevent approach of other molecules to the binding sites nearby. The approach of ligands during their binding to the adjacent sites can be thermodynamically disadvantageous. In the limiting case, the binding of two ligand molecules to adjacent sites can be completely excluded (model with binding site exclusion) [23]. This model allows us to explain a relatively low saturation level of the binding sites on LPS with the long O-polysaccharide chains.

Specific features of the Ch-LM and Ch-HM conformations can also influence the manifestation of these effects. The Ch-LM molecule is rather flexible [16] and its conformation in solution presents a randomly coiled sphere, whereas the Ch-HM molecule is rather rigid and elongated. Due to the chain flexibility, the binding sites on chitosan can spatially displace one relative to another and lower steric obstacles at the binding of high-molecular-weight ligands that is impossible in the case of rigid elongated molecules of Ch-HM. This seems to explain our finding, that the number of binding sites per potential binding link in Ch-LM is higher than in Ch-HM.

This explanation is confirmed by transformation of the saturation curves according to the following equation as proposed in the review [28]:

$$[1 - Q]C/Q = f(C).$$

The tangent of the inclination angle of this curve denoted as κ and equal to zero indicates the absence of cooperativity. In our case $\kappa = 0.5$ and 0.125 for the Ch-HM complexes with LPS-2 and LPS-3, respectively, which suggests negative cooperativity.

Thus, our data have shown that LPS-2 and LPS-3 bind to Ch-HM with negative cooperativity.

We observed that the increase in the O-chain length was associated with decrease in the number of binding sites on the endotoxin per link of chitosan. Thus, the increase in the molecular weight of LPS caused by elongation of the O-specific polysaccharide, from 6.2 to 23.5 kD, was associated with a decrease in the number of binding sites on the endotoxin molecule from 0.27 to 0.03 as calculated per monomeric link of chitosan. This find-

ing can be explained by a partial shielding by the long O-chains of sites on the LPS molecule, which are responsible for the binding (such as negatively charged groups of the core and lipid A) and can also provide spatial difficulties for the ligand binding to the adjacent sites.

Because of aggregated state of LPS, their negative charges are not easily available for amino groups; therefore, many more positive charges are required for saturation than for a pure compensation of the charge [8]. Note that the binding of Ch-LM with ^{125}I-labeled LPS-3 occurred with positive cooperativity. The cooperative interaction was also observed by us for the binding of immobilized membrane proteins, porins, with LPS possessing long O-polysaccharide chains [29].

The cooperativity is likely to be due to immobilization of chitosan. This leads to fixation of a number of chitosan molecules in a limited space and can be favorable for interaction of the bound ligand with the neighboring chitosan molecule through both ligand–ligand interaction (production of hydrogen bonds between the closely located O-polysaccharides of the bound ligands) and ligand–acceptor binding (production of the bond between the free valent group of the ligand and the adjacent molecule of chitosan). The positive cooperativity of LPS-3 binding with Ch-LM can be provided by the carbohydrate–carbohydrate interaction of the long O-chains of LPS bound with chitosan. Such type of intermolecular interaction occurs on aggregating of LPS possessing long O-specific chains [30]. The approach of chitosan-bound LPS molecules, which ensures contact necessary for such an interaction, in this experiment seems to be obtained due to long O-chains of LPS and specific features of the Ch-LM conformation.

The numbers of LPS-3 binding sites for Ch-LM determined by different methods are similar. Thus, the study on the LPS interaction with chitosan by the approach based on the dye replacement seems to take into account all bound ligands.

It should be emphasized that the data presented in this work were obtained on dilute solutions of the polymers (LPS concentration <0.1 mg/ml). In this case, in the saturation point, on average, 9-11 glucosamine residues are present per mole of LPS-2, depending on the molecular weight of the chitosan. However, it was earlier shown [9] that in concentrated solutions (LPS concentration 1 mg/ml) there were, on average, 200 glucosamine residues per mole LPS during the binding of the same LPS with chitosan.

Thus, the lower is the concentration of LPS, the higher is its amount bound to chitosan during complexing. Such a concentration dependence of stoichiometry of the complexes seems to be determined by the aggregative state of LPS, which depends on its concentration in the solution [31]. This is supported by our data on the crucial significance of the aggregative state of LPS for its binding with chitosan [9].

Thus, the interaction affinity and stoichiometry of the LPS–chitosan complexes are essentially determined by the structure of LPS and their concentration in the reaction mixture. Increase in the length of the carbohydrate chains of LPS strengthens the interaction of the complex components and lowers the amount of the bound LPS. We have earlier established that the binding of LPS with chitosan decreases its toxicity [32]. Varying the stoichiometry and composition of the LPS–chitosan complexes, we plan to study the effects of these factors on the biological activity of endotoxins.

REFERENCES

1. Morrison, D. C., Kirikae, T., Kirikae, F., Lei, M.-G., and Vukajlovich, S. W. (1994) in *Bacterial Endotoxins: Basic Science to Anti-sepsis Strategies* (Levin, J., Deventer, S., Poll, T., and Sruk, A., eds.) Wiley-Liss, N. Y., pp. 3-16.
2. Moore, R. A., Bates, N. C., and Hancock, R. E. W. (1986) *Antimicrob. Agents Chemoter.*, **29**, 496-500.
3. Brandenburg, K., Koch, M. H. J., and Seydel, U. (1998) *Eur. J. Biochem.*, **258**, 686-695.
4. Ohno, N., and Morrison, D. C. (1989) *J. Biol. Chem.*, **264**, 4434-4441.
5. Ohno, N., and Morrison, D. C. (1989) *Eur. J. Biochem.*, **186**, 621-627.
6. Yermak, I. M., Naberezhnyk, G. A., Drozdov, A. L., Solov'eva, T. F., and Ovodov, Yu. S. (1994) *J. Biochem. Organization*, **1**, 295-304.
7. Raetz, C. R. H. (1990) *Ann. Rev. Biochem.*, **59**, 129-170.
8. Brandenburg, K., Arraiza, M. D., Lehwark-Ivetot, G., Moriyon, I., and Zahringer, U. (2002) *Termochim. Acta*, **394**, 53-61.
9. Davidova, V. N., Yermak, I. M., Gorbach, V. I., and Solov'eva, T. F. (1999) *Biol. Membr. (Moscow)*, **16**, 42-49.
10. Davidova, V. N., Yermak, I. M., Gorbach, V. I., Krasikova, I. N., and Solov'eva, T. F. (2000) *Biochemistry (Moscow)*, **65**, 1082-1090.
11. Galanos, C., and Luderitz, O. (1969) *Eur. J. Biochem.*, **9**, 245-249.
12. Westphal, O., Luderitz, O., and Bister, F. I. Z. (1952) *Naturforsch.*, **7b**, 148-155.
13. Brade, H., and Galanos, C. (1982) *Eur. J. Biochem.*, **122**, 233-237.
14. Krasikova, I. N., Gorbach, V. I., Solov'eva, T. F., and Ovodov, Yu. S. (1978) *Eur. J. Biochem.*, **89**, 287-289.
15. Tkatch, B., and Stachelin, T. (1983) in *Immunology: Investigation Methods* (Lefkovitz, I., and Pernis, B., eds.) [Russian translation], Mir, Moscow, pp. 39-71.
16. Davidova, V. N., Yermak, I. M., Gorbach, V. I., Drozdov, A. L., and Solov'eva, T. F. (2000) *Biofizika*, **45**, 641-647.
17. Varfolomeev, S. D., and Gurevich, K. G. (1999) in *Biokinetics* [in Russian], Fair-Press, Moscow, pp. 395-396.
18. Dische, Z. (1967) in *Approaches in Carbohydrate Chemistry* (Kochetkov, ed.) [Russian translation], Mir, Moscow, pp. 48-52.
19. Thakur, L. D., Jaffe, M. L., and Rodbad, D. (1980) *Analyt. Biochem.*, **107**, 719-725.
20. Glazunov, V. P., and Gorbach, V. I. (1999) *Bioorg. Khim.*, **25**, 216-219.

21. Hannecart-Pokorni, E., Dekegel, D., Depuydt, F., and Dirkx, J. (1970) *Biochim. Biophys. Acta*, **201**, 155-156.
22. Mathison, J. C., Tobias, P. S., Wolfson, E., and Ulevitch, R. J. (1992) *J. Immunol.*, **149**, 200-206.
23. Cantor, Ch., and Shimmel, P. (1985) *Biophysical Chemistry* [Russian translation], Mir, Moscow.
24. Knirel, A. Yu. (1985) in *Progress in Chemistry of Carbohydrates* (Torgov, I. V., ed.) [in Russian], Nauka, Moscow, pp. 54-76.
25. Zavodinskii, V. G., Gnidenko, A. A., Davidova, V. N., and Yermak, I. M. (2003) *Chemistry and Computer-Aided Modeling. Butlerov Reports*, **2**, 12-13.
26. Augusto, L. A., Li, J., Synguelakis, M., Johansson, J., and Chaby, R. (2002) *J. Biol. Chem.*, **277**, 23484-23492.
27. Seydel, U., Brandenburg, K., and Rietschel, E. T. (1994) *Prog. Clin. Biol. Res.*, **388**, 17-30.
28. Acerenza, L., and Mizraji, E. (1997) *Biochim. Biophys. Acta*, **1339**, 155-166.
29. Naberezhnykh, G. A., Khomenko, V. A., Krasikova, I. N., Kim, N. Yu., and Solov'eva, T. F. (1996) *Bioorg. Khim.*, **22**, 671-677.
30. Peterson, A. A., Haug, A., and McCroarty, E. J. (1986) *J. Bacteriol.*, **165**, 116-122.
31. Yermak, I. M., Yadykina, G. M., Solov'eva, T. F., and Ovodov, Yu. S. (1985) *Biofizika*, **33**, 288-291.
32. Yermak, I. M., Gorbach, V. I., Polyakova, A. M., Astrina, O. S., Luk'yanov, P. A., Solov'eva, T. F., Maleev, V. V., and Ovodov, Yu. S. (1994) *Biol. Membr. (Moscow)*, **11**, 496-500.