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^{31}P NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF LIPOPOLYSACCHARIDES FROM *PSEUDOMONAS AERUGINOSA*

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Summary

Intact lipopolysaccharide antigens isolated from seven different immunotypes of *Pseudomonas aeruginosa* have been examined by ^{31}P-NMR spectroscopy. These macromolecular complexes contain phosphorus covalently attached to the carbohydrate residues present in the lipid A moiety and the 'core' oligosaccharide region. The spectral signals for various ortho- and pyrophosphoric esters were observed. All phosphate groups appeared to be monoesterified. Certain shifts characteristic for phosphate diester groups, observed in lipopolysaccharide complexes from other Gram-negative bacteria, were absent. Furthermore, no evidence was found to indicate that phosphate groups are involved in the covalent linkage of individual lipopolysaccharide complexes to form dimers or trimers.

Introduction

The lipopolysaccharide complex is a ubiquitous component of the outer membrane of Gram-negative bacteria. The generalized model for its structure, based upon studies on the smooth ('wild-type') strains of *Salmonella* [1,2], comprises three main components (side-chain, core, and lipid A) that are covalently linked. Several review articles have described the chemical composition and immunological properties of lipopolysaccharide complexes isolated from a wide variety of bacteria in the family Enterobacteriaceae [2—6].

Lipopolysaccharides from seven immunotypes of *Pseudomonas aeruginosa* (in the classification scheme of Fisher et al. [7]) have been isolated and subjected to analytical characterization [8,9]. Each of these complexes contains approx. 3% (w/w) phosphorus [8]. Most enterobacterial lipopolysaccharides contain [10] about seven phosphate residues distributed in the inner core region and in lipid A. However, it has been pointed out lipopolysaccharides from *Ps. aeruginosa* may contain ten or more phosphate residues in a single complex [11—13].

Analyses by ^{31}P-NMR spectroscopy of the phosphate residues in lipopolysaccharides of *Salmonella* and *Escherichia coli* have appeared in the literature [10,14—16]. Definite similarities are noted between the results of the experiments described here and those conducted on different forms of lipopolysaccharide complexes isolated from *Salmonella* and *E. coli*. Phosphate monoester and monoesterified pyrophosphate residues were found here in all seven *Ps. aeruginosa* lipopolysaccharide complexes. Preliminary evidence also suggests that the presence of pyrophosphoryl ethanolamine may be variable in lipopolysaccharides of *Ps. aeruginosa*. None of the spectra indicated the individual lipopolysaccharide complexes to be crosslinked by phosphate diester or pyrophosphate diester bonds. Similar observations have already been made for lipopolysaccharides of *Salmonella* [10].

Additional phosphate groups in lipopolysaccharides of *Ps. aeruginosa* appear to be present in the form of phosphate monoesters. These phosphate residues must occur in the core region, as only a finite number of phosphate groups can occur in the lipid moiety (especially as monoesters).

Materials and Methods

Ps. aeruginosa lipopolysaccharide antigens (Fisher immunotypes 1—7) were supplied by Parke, Davis and Co., Detroit, MI. The isolation procedure used has already been described [17]. *E. coli* alkaline phosphatase (Type III ®) was obtained from Sigma Chemical Co.

^{31}P-NMR spectra were recorded at 36.43 MHz with a Bruker HX-90 spectrometer in the Fourier-transform mode. The field was locked on the deuterium resonance (15.40 MHz) of the solvent (75% ^2H_2O) at probe temperature. The sweep width was 5000 Hz with an aquisition time of 0.8 s and a pulse width of 4 μs. Between 10 000 and 20 000 scans were accumulated for each spectrum. All spectra were broad-band proton-decoupled. Phosphorus chemical shifts are in parts per million (ppm) relative to the ^{31}P resonance of an external standard, 85% aqueous orthophosphoric acid.

In a typical experiment, 50 mg lipopolysaccharide was suspended in 2 ml 99.8% D_2O/0.2 ml 0.1 M EDTA/4 drops triethylamine. The apparent pH of the final solution was 10.5. The suspension was then placed in a sonic bath for 15 min. The solution was then filtered into an NMR tube (12 × 180 mm), and the spectra were recorded.

In the alkaline phosphatase experiment, 50 mg lipopolysaccharide was suspended in 2 ml 99.8% ^2H_2O and 4 drops of triethylamine. The pH was then adjusted to 8.0 with 0.1 N HCl, and 75 μl alkaline phosphatase in 2.6 M $(NH_4)_2SO_4$ (2 mg protein/ml; 44 units/mg protein) was added. After 30 min, 0.2 ml 0.1 M EDTA was added and the pH was raised to 10.5 with additional triethylamine.

Results

For each lipopolysaccharide examined, there were three major groups of signals observed, near −3 ppm, 5 ppm, and 10 ppm; each spectrum also showed a signal at 0 ppm. Typical spectra of 'intact' lipopolysaccharide complexes

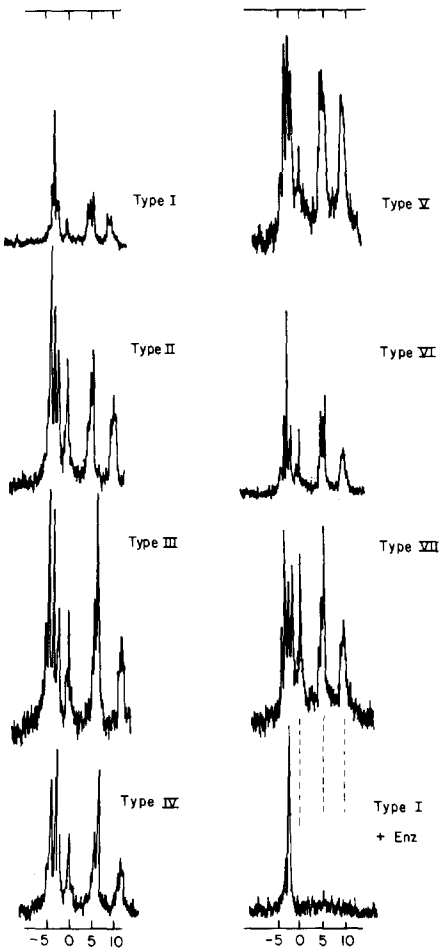

Fig. 1. ^{31}P-NMR spectra of 'intact' lipopolysaccharide complexes from seven immunotypes of *Ps. aeruginosa*, and of immunotype one lipopolysaccharide after treatment with alkaline phosphatase (Enz). Chemical shifts are in ppm from external phosphoric acid.

from all seven immunotypes are given in Fig. 1 (see also Table I).

As the nuclear Overhauser enhancement of proton-decoupled signals may be different for variously linked phosphorus nuclei, no attempt was made to evaluate the integrated peak areas. The signals at 5 ppm and 10 ppm could arise from the β-phosphorus and α-phosphorus atoms of a pyrophosphoric monoester, respectively. However, the qualitative ratio of the areas at 5 ppm and 10 ppm is 2 : 1 in most of these spectra (whereas it should be 1 : 1). At the pulse angle used in these experiments, this result could be anticipated [16]. The increase in intensity could also arise from the presence of orthophosphoric diester, which could have a shift at +5 ppm.

To investigate the possible presence of phosphate diesters, alkaline phosphatase was added to the lipopolysaccharide complex isolated from *Ps. aeruginosa* immunotype one. This resulted in the complete removal of all phosphate signals higher than 5 ppm, indicating that these signals arose from pyrophos-

TABLE I

^{31}P CHEMICAL SHIFTS OBSERVED FOR *Ps. AERUGINOSA* LIPOPOLYSACCHARIDE COMPLEXES *

Immunotype	Nonglycosidic				Free phosphate	Glycosidic	Other phosphate	β-pyro-phosphate	α-pyro-phosphate
	−4.7	−4.3	−3.8	−3.2	−2.5	−2.2	−0.1	+5.5	+10.5
One	s	s	s	s	s	s	s	m	m
Two	—	s	s	s	—	s	s	m	m
Three	s	s	s	s	s	s	s	m	m
Four	—	—	s	s	—	s	s	m	m
Five	s	—	s	s	—	s	s	m	m
Six	—	—	s	s	s	s	s	m	m
Seven	s	—	s	s	—	s	s	m	m
One + enzyme	—	—	s	—	s	—	—	—	—

* s, singlet; m, multiplet; —, absent.

phoric monoesters. Alkaline phosphatase is sometimes contaminated with a small amount of pyrophosphatase. However, control experiments, treating adenosine 5′-monophosphate and nicotinamide adenine dinucleotide (separately), with the alkaline phosphatase, followed by adenosine deaminase (to produce inosine), showed that the alkaline phosphatase was active only on phosphoric monoesters. Therefore, it was concluded that all the signals at 5 and 10 ppm arose from the β and α phosphate, respectively, of a pyrophosphate monoester (at least in immunotype one). As there are more than two peaks at +5 and +10 ppm, there must be at least two different pyrophosphate groups.

Most of the other phosphate signals, suspected to arise from phosphate monoesters, were also removed by the alkaline phosphatase to leave a single peak at −2.6 ppm. At a pH of 10.5, this signal corresponds to free phosphate. However the peak at −3.8 ppm was not affected by the enzyme. This phosphate group may be attributed to the phosphate monoester attached to O-4′ of the 2-amino-2-deoxy-D-glucose disaccharide of the lipid A moiety. The same phosphorus signal has already been observed and assigned in lipopolysaccharides of *Salmonella* [10] and *E. coli* [16]. This signal appears in the spectra of all seven lipopolysaccharide complexes studied in this laboratory.

The other phosphate monoester groups give signals at −4.7, −4.3 and −3.2 ppm. All of these esters were susceptible to cleavage by the enzyme, and most probably are present in the inner core oligosaccharide [10]. The signals at −4.7 and −4.3 ppm were variably present in these lipopolysaccharide complexes. However the one at −3.2 ppm was present in all of the *Ps. aeruginosa* immunotypes. (see Table I)

The signal at −2.2 ppm has been assigned to the C-1 substituent of the 2-amino-2-deoxy-D-glucose disaccharide of the lipid A moiety [10,16] (i.e. it is the glycosyl phosphate). It is also cleaved by alkaline phosphatase. The question of α or β anomeric configuration may not be readily resolved by ^{31}P-NMR [16].

The only phosphate group of which the assignment remains obscure is the one giving rise to the signal at −0.1 ppm. Related studies have suggested that it corresponds to a monoester, monoanion phosphate residue [18]. It may be present in the 'core' or in the lipid A moiety.

Discussion

The overall architecture of the lipopolysaccharides complex of *Ps. aeruginosa* has proved to be quite similar to that of other Gram-negative bacteria [3]. These immunotypes appear to have a (1 → 6)-linked, 2-amino-2-deoxy-D-glucose disaccharide backbone in the lipid A moiety, which is *O*- and *N*-substituted with hydroxy and nonhydroxy fatty acids [19,20].

Comparing these spectra with those published for lipopolysaccharide of other Gram-negative bacteria establishes that all have phosphate monoesters in the oligosaccharide of the inner core region of the lipopolysaccharide, the reducing end of the 2-amino-2-deoxy-D-glucose disaccharide is substituted with at least one phosphate, and all contain pyrophosphate esterified to either the core or the lipid in a variable manner.

One signal not appearing in any of these spectra is one at 2.5 ppm. In *Salmonella*, such a signal corresponds to the phosphate diester linkage between 4-amino-4-deoxy-L-arabinose and the O-4' of the 2-amino-2-deoxy-D-glucose disaccharide of the lipid [10]. This arabinose residue has not thus far been reported in *Ps. aeruginosa*, possibly because of its extreme lability. However, the present studies suggest that the aminopentose is not present in *Ps. aeruginosa*.

The presence of phosphoryl ethanolamine has been established unequivocally in the lipopolysaccharides of some strains of *Ps. aeruginosa* [13,21]. However, it has also been proposed that it is most probably a variable constituent that may be added or omitted to modify the overall negative surface charge of Gram-negative bacteria [10]. This could naturally be a function of bacterial growth conditions. Clearly, the presence of an esterified 2-aminoethyl pyrophosphate is ruled out in the lipopolysaccharide of immunotype one, as it would be resistant to alkaline phosphatase. Also it would give rise to a strong quartet at 10 ppm, which is not observed in the spectra of lipopolysaccharide from any of the immunotypes. The possibility of any metaphosphate is also ruled out, as it would give a signal upfield from 10 ppm. This contrasts with an earlier report that *Ps. aeruginosa* may be able to produce 2-aminoethyl metaphosphate [13]. 2-Aminoethyl triphosphate was isolated from a strain of *Ps. aeruginosa* by degradative means. The lipopolysaccharide complexes in these two studies were isolated from the whole bacteria by different procedures, but a 2-aminoethyl metaphosphate esterified to part of the lipopolysaccharide complex should have remained intact. As no ^{31}P-NMR signal for metaphosphate was observed in these 'intact' lipopolysaccharide complexes, metaphosphate is unlikely to be a normal constituent of the lipopolysaccharide complex.

The last question to be addressed is the role of phosphorus in maintaining the overall size of the lipopolysaccharide complex. Another study in this laboratory has determined the molecular weights of the side-chain polysaccharides of these lipopolysaccharide complexes to be about 15 000 [22]. Estimations of the molecular weight of the core and lipid have given values of 2000 and 3000, respectively [23]. The total of these would suggest an overall molecular weight of about 20 000. However, the intact lipopolysaccharide complexes used in this study are eluted in the excluded volume from a column of Sephadex G-100, indicating a molecular weight of more than 100 000, or at least 5-times larger than the sum of the individual components (namely side-chain + core + lipid). It was first proposed, from ultracentrifugation data, that this was because of phosphate groups crosslinking the lipid or core polysaccharide of individual lipopolysaccharide units to form dimers, trimers, and so on [24—27]. However, the present and previous ^{31}P-NMR studies have not demonstrated any evidence for phosphate groups playing a role in crosslinking individual lipopolysaccharide components [10,16]. In addition, a recent sedimentation-equilibrium study has shown that the lipopolysaccharide from *Salmonella* dissociates upon the removal of divalent cations and with the addition of a surfactant (sodium deoxycholate) [23]. The conclusion seems to be individual lipopolysaccharide units are held together by ionic hydrophobic interactions [10,23].

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