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Configurations of Glycosidic Phosphates of Lipopolysaccharide from *Salmonella minnesota* R595†

Michael Batley,* Nicolle H. Packer, and John W. Redmond

ABSTRACT: The anomeric configurations of the reducing terminal glucosamine and 4-amino-4-deoxy-L-arabinose phosphates in lipopolysaccharide from *Salmonella minnesota* R595 have been determined by nuclear magnetic resonance. Chemical shifts for the anomeric protons were obtained by selective decoupling of the phosphorus spectrum and proton–proton coupling constants by polarization transfer from protons to phosphorus. In both cases, the phosphate is attached to the sugar in an axial orientation.

Considerable progress has been made in the determination of the chemical nature of LPS[1] from Gram-negative bacteria (Galanos et al., 1977). The lipidic subunit of the molecule, known as lipid A, is similar in both *Escherichia coli* K-12 (Rosner et al., 1979a,b,c) and *Salmonella minnesota* (Gmeiner et al., 1969; Mühlradt et al., 1977), but some aspects of the structure remain undetermined. In particular, the anomeric configuration is known for only one of the three sugars in lipid A. These configurations will play an important part in determining the shape of the molecule and its ability to pack into bilayer membranes. The information is also of importance to workers attempting to synthesize the minimal active subunit of the endotoxin (Inaga et al., 1981; Nashed & Anderson, 1981).

Strain R595 of *S. minnesota* is a deep rough mutant (Gmeiner et al., 1969), and LPS from it has a side chain consisting only of a branched trimer of 3-deoxy-D-*manno*-octulosonic acid attached at the 3′ position of lipid A (Figure 1). The lipid A itself consists of two β1→6-linked D-glucosamine residues, with approximately five ester-linked and two amide-linked fatty acids. The 1 and 4′ positions of the disaccharide are phosphorylated. The 1 substituent is mostly

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[1] Abbreviations: LPS, lipopolysaccharide; NMR, nuclear magnetic resonance; EDTA, disodium ethylenediaminetetraacetate; KDO, 3-deoxy-D-*manno*-octulosonic acid.

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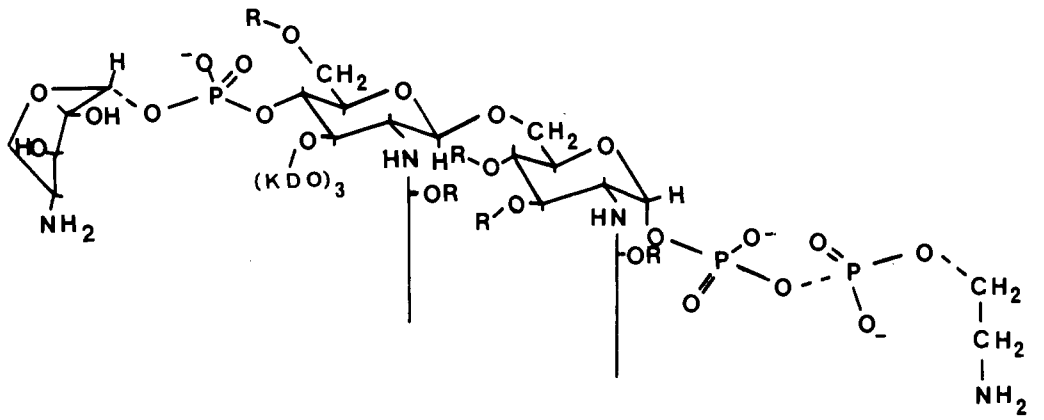

FIGURE 1: Molecular structure of LPS from *S. minnesota* R595: R = fatty acyl or H. Dashed lines indicate partial substitution.

phosphomonoester, but small amounts of pyrophosphoryl monoester and diester are also present. The 4′ position carries either monoester or 4-amino-4-deoxy-L-arabinosyl diester (Mühlradt et al., 1977). The amount of diester in freshly prepared LPS is probably much greater than was previously thought (M. Batley and J. W. Redmond, unpublished results).

The use of deoxycholate to disperse de-*O*-acyl-LPS, produced by treatment with hydrazine, results in phosphorus-31 spectra with greatly reduced line widths (Batley et al., 1981). The improved resolution has made possible the determination by phosphorus NMR of the previously unknown configurations of the anomeric phosphates.

Materials and Methods

Salmonella minnesota strain R595 was grown in shake cultures in nutrient broth (Difco, Detroit, MI) overnight at 37 °C to stationary phase. The yield was approximately 0.4 g of dry mass/L. The harvested cells were washed twice with water and dried by repeated washing with acetone.

LPS was obtained from dried cells by extraction with phenol–chloroform–petroleum ether (Galanos et al., 1969). The yield was 2.5%.

Ester-linked fatty acids were removed from LPS by treatment with hydrazine. LPS (50 mg) was dispersed in anhydrous hydrazine (1 mL) in a sealed tube and heated in boiling water for 30 min. The tube was cooled in ice and the mixture heated cautiously with excess acetone. After removal of hydrazine by repeated evaporation with acetone under reduced pressure, the residue was extracted 3 times with acetone to remove fatty acyl hydrazides. The acetone-insoluble material was dispersed in water and purified either by chromatography on a Sephadex G-25 column with a pyridinium acetate buffer, pH 5.3, or by ultrafiltration in an Amicon cell (Model M-3) fitted with a YM-10 membrane. The dispersion was filtered under nitrogen pressure, with five changes of water (3 mL); then the contents of the cell were removed and freeze-dried. De-*O*-acyl-LPS was N-acetylated as described before (Redmond, 1978).

NMR spectra were obtained on a Varian XL-200 spectrometer operating at 81 MHz for phosphorus and 200 MHz for protons. The spectra were recorded with a deuterium field lock, and the temperature was maintained at 45 °C. The decoupling power was kept low to avoid sample heating and consequent loss of resolution. Proton spectra were referenced to sodium 3-(trimethylsilyl)propanesulfonate in D_2O, measured separately because the presence of surfactant precluded direct observation of proton spectra. We have found that chemical shift measurements relying in this way on the field lock are accurate to ±2 Hz provided the temperature is controlled.

Phosphorus spectra were referenced to 85% phosphoric acid in a spherical sample tube. The spectra were recorded by using a cylindrical sample as a secondary reference and the results corrected for sample shape by subtracting 0.73 ppm (Batley & Redmond, 1982). Results obtained with cylindrical tubes perpendicular to the magnetic field are seldom corrected for the difference between the diamagnetic susceptibilities of water and phosphoric acid. For a comparison of such results with the present work, 0.37 ppm should be added to the reported values. Chemical shifts downfield from phosphoric acid are positive.

The samples for spectroscopy were prepared by dispersing 10–20 mg of de-*O*-acyl-LPS in 2 mL of D_2O with the aid of 1 or 2% w/v surfactant. Sufficient EDTA was added to make the solution 5 mM, and the pH was adjusted with sodium hydroxide solution. No corrections were made for the effect of D_2O on the pH electrode on the basis of the customary legerdemain of assuming that there may be compensating effects on pK_a values.

Results

Proton–Phosphorus Spin–Spin Coupling. The surfactants deoxycholate and taurodeoxycholate were equally effective at dispersing de-*O*-acyl-LPS, giving line widths of 0.6 Hz for the sharpest lines in the phosphorus spectra. Sodium dodecyl sulfate produced lines that were only slightly broader. Without the detergents the line width was 3.9 Hz.

The improved resolution revealed apparent heterogeneity. Three signals were observed, all with the chemical shift expected for the glycosidic 1-phosphate, but with slightly differing pK_a values. Two of the signals were relatively small, and their detailed assignments will be discussed elsewhere (M. Batley and J. W. Redmond, unpublished results).

It was also possible to resolve four-bond coupling between phosphorus and the proton at the 2 position of glucosamine. Figure 2 shows the proton-coupled ^{31}P spectrum of de-*O*-acyl-LPS. The doublet at 0.2 ppm is the 4′ monoester, the doublet of doublets at −1.5 ppm the 1 monoester, and the broad triplet at −2.1 ppm is the 4′ diester.

The coupling constants for the 1-phosphate are recorded in Table I. The coupling constant for the 4′ monoester is pH dependent as shown in Table II. Selective proton decoupling was used to measure the spin–spin coupling constants for the diester. At pH 7.85, H4′ splits the diester resonance by 6.8 ± 0.2 Hz, and the anomeric proton of the aminoarabinose substituent has a 6.4 ± 0.1 Hz coupling constant.

Chemical Shifts of Anomeric Protons. Selective proton irradiation with a low radio frequency field strength (γB_2 = 110 Hz) was used to determine the chemical shift of the

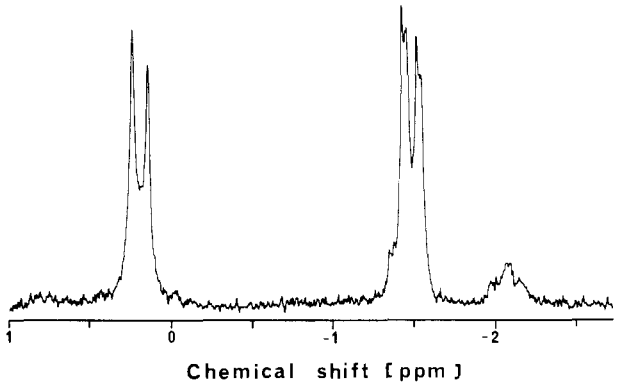

FIGURE 2: Proton-coupled ^{31}P spectrum of de-*O*-acyl-LPS with 5 mM EDTA and 1.5% sodium taurodeoxycholate, pH 5.5, 45 °C.

Table I: ^{1}H Chemical Shift and Coupling Constants for De-*O*-acyl-LPS and Model Compounds

compound	pH	σ^a	$J_{1,2}$	$J_{P,1}$	$J_{P,2}$
de-*O*-acyl-LPS	5.5			7.2	1.85
	7.85	5.38	2.4	7.5	1.3
N-acetyl-α-D-glucosamine 1-phosphate[b]		5.34	2.7	7.5	
α-D-glucose 1-phosphate[c]	4.0	5.85	3.5	7.0	2.6
	8.0	5.44	3.4	7.5	1.8

[a] The chemical shifts are expressed as parts per million from internal 3-(trimethylsilyl)propanesulfonate. [b] Khorlin et al., 1970. [c] O'Connor et al., 1979.

Table II: ^{31}P-^{1}H Spin–Spin Coupling Constants for 4′ Monoester

pH	$J_{P,H}$[a]	population of rotamer I[b] (%)
5.5	7.7	27
6.9	6.4	21
7.9	5.6	17
9.0	4.8	13

[a] Values are accurate to ±0.1 Hz. [b] See Discussion for the definition of rotamer geometry.

anomeric protons. As shown in Figure 3, it was possible to determine the chemical shift to within 0.05 ppm. The chemical shift for H1 of the glucosamine subunit is given in Table I, while those for aminoarabinose H1 and glucosamine H4′ are 5.57 ± 0.05 ppm and 3.9 ± 0.1 ppm, respectively.

Indirect Detection of Proton–Proton Splitting. The presence of the large amounts of surfactant prevents direct observation of proton spectra. The H1–H2 spin–spin coupling constants, which are important for the assignment of anomeric configuration, were therefore measured indirectly by polarization transfer. The validity of the method was first demonstrated with α-D-glucose 1-phosphate.

The spectra of protons spin coupled to phosphorus nuclei can be detected by transfer of spin population difference to the phosphorus, followed by observation of the phosphorus signal. The transfer may be performed with the INEPT pulse sequence (Morris & Freeman, 1979), suggested originally as a means of increasing signals from insensitive nuclei. Since the sequence employs delays that are inversely proportional to the proton–phosphorus coupling constant, $J_{P,H}$, it is possible, in favorable circumstances, to obtain separate spectra for protons with different coupling constants. The spectra of H1 and H2 of glucose 1-phosphate were separated successfully.

The pulse sequence employed was composed of three sections: preparation, polarization transfer, and refocusing. The

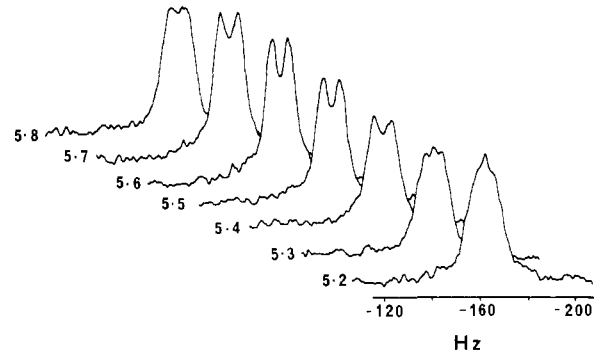

FIGURE 3: Selective proton decoupling of the ^{31}P spectrum of the 4′-diester signal from de-*O*-acyl-LPS, with 5 mM EDTA and 1% sodium taurodeoxycholate, pH 7.8, 45 °C. The proton chemical shift, in ppm, used for irradiation is indicated beside each spectrum.

pulses in each part of the sequence may be represented as preparation

$$(\pi/2)_{H,x} - \tau/2 - \pi_H - \tau/2$$

polarization transfer

$$1/(4J) - \pi_H,\pi_P - 1/(4J) - (\pi/2)_{H,y}(\pi/2)_{P,x}$$

refocusing

$$1/(4J) - \pi_H,\pi_P - 1/(4J) - \mathrm{AT(decouple)}$$

Phase cycling was used to remove normal phosphorus magnetization (Morris & Freeman, 1979). The proton spectrum is obtained by allowing the proton magnetization to precess in the *xy* plane for various times before polarization transfer. The intensity of the phosphorus signal is then a measure of the *x* component of proton magnetization at the end of the preparation period. A two-dimensional spectrum may be obtained by Fourier transformation of the phosphorus intensity as a function of the evolution time, τ. The application of the proton π pulse in the middle of the preparation period refocuses any chemical shift or phosphorus–proton coupling information, leaving only proton–proton splitting displayed along the second frequency axis. Elimination of the chemical shift is necessary for the later experiments where the signals are too weak to permit Fourier transformation in the second domain.

Two-dimensional spectra from α-D-glucose 1-phosphate obtained in this way are shown in Figure 4. By using the observed values of $J_{P,H1}$ = 7.2 Hz and $J_{P,H2}$ = 1.8 Hz for J in the pulse sequence, it was possible to transfer polarization selectively from the two protons. The small shoulders on the inside of the central peaks for the H2 multiplet are due to the superimposition of some intensity from H1, because of failure to match $J_{P,H1}$ precisely. Comparison of projections of the above spectra onto the proton frequency axis with the normal proton spectrum is made in Figure 5. The spectra are equivalent except that phosphorus–proton splitting is absent from the former.

Glucosamine H1 Spectra. The spectra of de-*O*-acyl-LPS required acquisition times of 12 h or more. Despite the theoretical prediction that noise in two-dimensional spectra should be dispersed over the two dimensions, such spectra require considerably longer accumulation times in practice. Consequently, spectra were obtained for a limited number of delay times, τ, and the phosphorus intensities were fitted by a nonlinear least-squares method (Margenau & Murphy, 1956) to an equation of the form

$$I = I_0 \cos(\pi J_{H,H} t) \exp(-\pi w t)$$

This is the behavior expected for a doublet with a splitting of

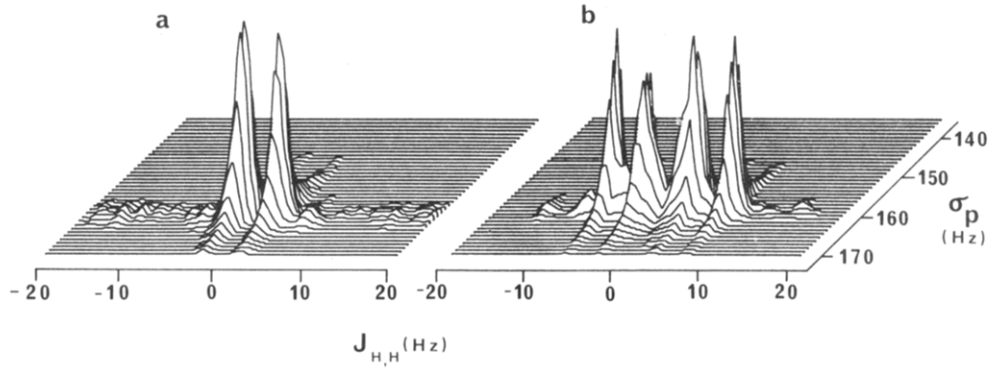

FIGURE 4: Proton–proton coupling vs. phosphorus chemical shift for α-D-glucose 1-phosphate in D_2O: (a) spectrum of H1 selected using $J_{P,H}$ = 7.2 Hz; (b) spectrum of H2 selected using $J_{P,H}$ = 1.8 Hz.

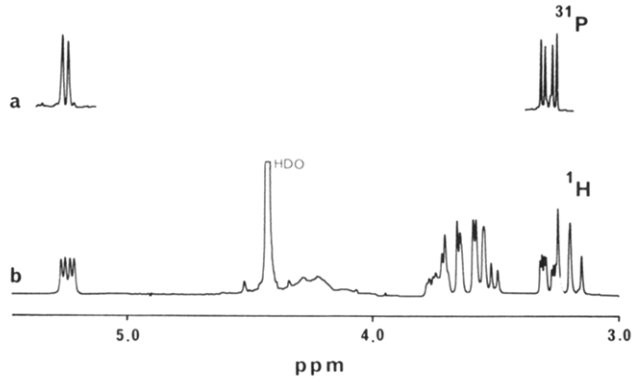

FIGURE 5: (a) Projection of ^{31}P two-dimensional spectra of α-D-glucose 1-phosphate onto the proton spin–spin splitting axis. (b) Normal ^{1}H spectrum of α-D-glucose 1-phosphate.

$J_{H,H}$ Hz and a line width of w Hz (assuming that the line shape is Lorentzian). The proton–proton interaction continues to affect precession during the polarization transfer period, so $t = \tau + 1/(2J_{P,H})$.

The phosphorus intensities for the anomeric phosphate on the reducing terminal glucosamine of de-*O*-acyl-LPS are plotted in Figure 6. The line of best fit corresponds to $J_{H1,H2}$ = 2.4 Hz and w = 1.9 Hz.

Aminoarabinose H1 Spectrum. The spectrum of the anomeric proton of aminoarabinose was more difficult to obtain. As shown in Figure 2, the diester peak is broad, and considerable intensity is therefore lost during polarization transfer pulse sequences. Furthermore, both H1 of the aminoarabinose and H4′ of the glucosamine can transfer intensity to the phosphorus. Both protons have similar coupling constants to the phosphorus, so J selection is not possible.

Separation of the H1 signal was achieved by saturation of the H4′ proton with selective irradiation prior to the usual preparation period. Refocusing of the phosphorus signal was omitted in order to minimize intensity loss due to the short T_2 values. A proton-coupled triplet with intensities of 1:0:−1 was thus obtained (Bodenhausen & Freeman, 1977). Intensities were measured from absolute value spectra for better signal-to-noise ratio, while the sign was obtained from phased spectra. Phasing was adjusted for the first spectrum and left unaltered throughout the series of experiments.

The field strength used for saturation was such that γB_2 = 300 Hz. For confirmation that this power was adequate, the anomeric proton magnetization was selectively inverted at the start of the pulse sequence. A soft proton pulse 6.0 ms long was applied at the frequency of H1. The frequency spectrum of this pulse has its second node 330 Hz to higher frequency, which is where the resonance frequency of H4′ is to be found. The phosphorus intensity with and without H1 inversion can

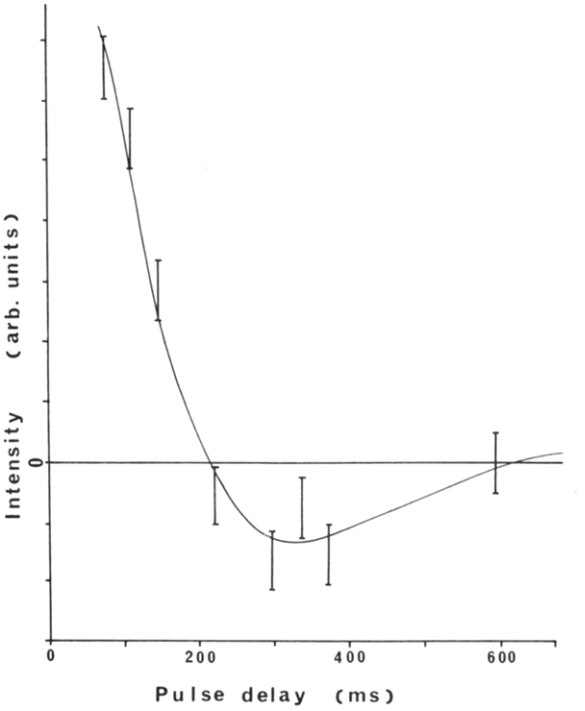

FIGURE 6: Intensity transferred to ^{31}P resonance of 1-phosphate of de-*O*-acyl-LPS as a function of proton preparation delay, t. The sample was dispersed in D_2O with 5 mM EDTA and 2% sodium deoxycholate, pH 8.0, 45 °C.

be used to measure the ratio of intensities transferred from H1 and H4′. As may be seen from Figure 7, the signal inverts and increases in intensity in response to inversion of H1. The major source of transferred intensity is therefore H1, and that from H4′ is opposite in sign. From the measured intensities it was concluded that the ratio of transferred magnetizations when τ = 0 is 1.00:−0.16. The small negative value for transfer from H4′ is in accord with our expectations. The proton resonance for H4′ should approximate a triplet with a splitting of 10–12 Hz, which is twice the $J_{P,H}$ value. If it were exactly double, the expected intensity transfer would be −0.33.

Efficient saturation of H4′ should increase the net intensity of the phosphorus signal from 0.84 to 1.00. It was found to increase to 0.98.

Spectra obtained for various values of the preparation delay time, t, are shown in Figure 8. The H1–H2 proton splitting in aminoarabinose is clearly closer to 3 Hz than to the 8 Hz expected for a diaxial coupling. The result of nonlinear least-squares analysis of the intensities is shown in Figure 9. A significantly better fit was obtained by omitting the point at t = 227 ms, and this is the line drawn in the figure. The parameters so obtained were $J_{H1,H2}$ = 3.5 Hz and w = 1.2 Hz.

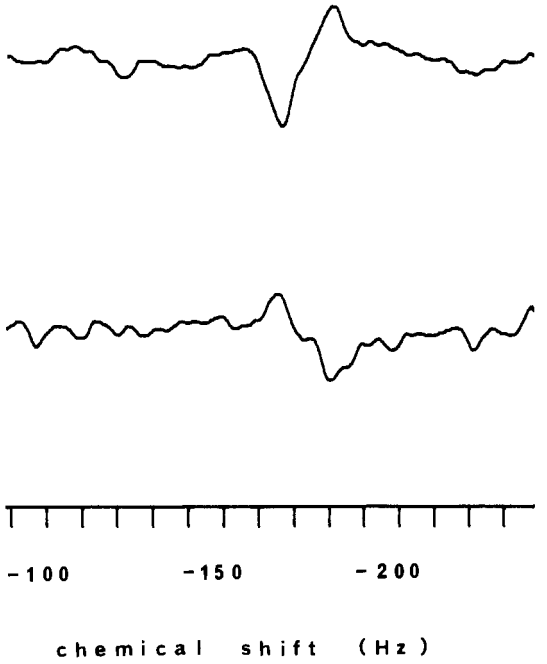

FIGURE 7: ^{31}P polarization transfer (a) with and (b) without selective inversion of H1 magnetization. The sample conditions are as in Figure 8.

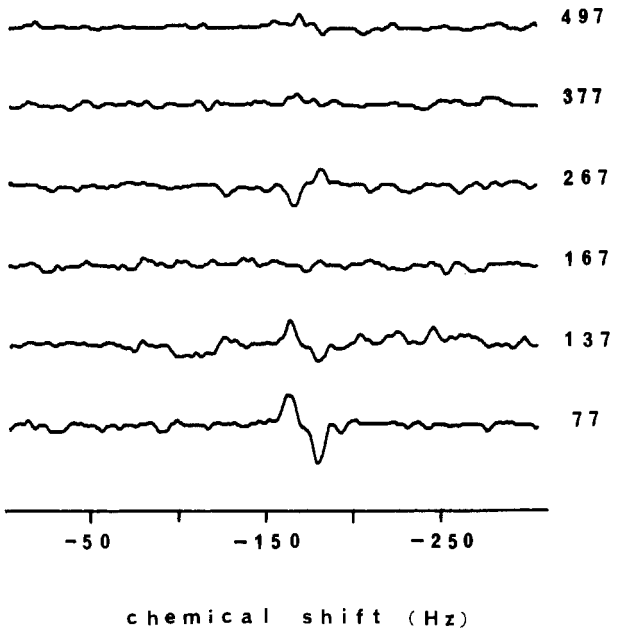

FIGURE 8: Proton-coupled ^{31}P polarization transfer spectra of 4′-diester signal from de-*O*-acyl-LPS in D_2O with 5 mM EDTA and 1% sodium taurodeoxycholate, pH 7.8, 45 °C. Proton preparation delay times in milliseconds are indicated beside each spectrum.

Although the precision of this result is less than that for the 1-phosphate, it is unlikely that J is in error by as much as 20%.

The line width of the proton signal may be compared with the width of the phosphorus peak. A phosphorus spectrum with H4′ selectively decoupled had a line width of 2.0 ± 0.1 Hz for the diester doublet. The line width of the inorganic phosphate resonance in the same sample was 0.7 Hz. This is therefore the lower limit for line-width contributions from magnetic field inhomogeneity and phosphorus spin–lattice relaxation. This leaves 1.3 Hz to be ascribed to the effect of the lifetime of H1 spin states (Abragam, 1961), which is in excellent agreement with the 1.2 Hz observed for the proton signal.

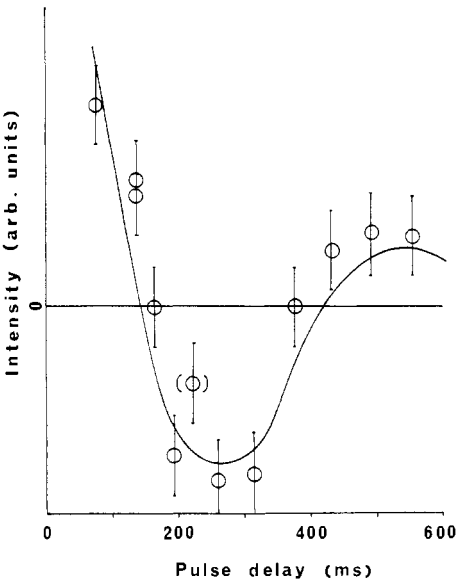

FIGURE 9: Intensity transferred to ^{31}P resonance of 4′ diester of de-*O*-acyl-LPS from H1 of aminoarabinose as a function of proton preparation delay, t. The sample conditions are as in Figure 8.

A similar treatment of the phosphorus line width obtained when selectively decoupling the other proton, H1, leads to an estimate of 2.9 Hz for the minimum line width of H4′, on the basis of the phosphorus width of 3.6 Hz. This demonstrates that, in keeping with intuition, the glucosamine is less mobile than the aminoarabinose. It also explains the breadth of the diester triplet. In the measurement of line width and the subsequent manipulations it has been assumed that all line shapes are Lorentzian.

N-Acetylation of Aminoarabinose. The proton spectrum of N-acetylated de-*O*-acyl-LPS contained a peak at 2.050 ± 0.003 ppm that was not present in unacetylated material. The peak width was 6 Hz, which is less than the 12 Hz observed for the fatty acyl signals, but much broader than that for free acetate (which was observed in small amounts despite extensive washing). Underlying resonances made integration difficult, but from the area of the new peak, it was estimated that 20–35% acetylation had been achieved.

Discussion

Anomeric Configuration of the Reducing Terminal Glucosamine. The H1–H2 spin-coupling constant is diagnostic for the anomeric configuration of the glucosamine phosphate of de-*O*-acyl-LPS, which is present as a pyranose ring (Gmeiner et al., 1971). The ring will adopt a $^4C_1$ conformation in order to place the four bulky substituents in equatorial positions. The small proton–proton scalar coupling is therefore indicative of an equatorial anomeric proton, and the reducing terminal sugar is therefore *N*-acyl-α-D-glucosamine 1-phosphate. The observed coupling constants are in good agreement with the corresponding parameters for *N*-acetyl-α-D-glucosamine 1-phosphate itself, as shown in Table I.

Further support for this assignment is provided by the chemical shift of H1. Anomeric protons in pyranose phosphates typically have chemical shifts of 4.8–5.1 ppm if they are axial, while equatorial protons appear in the range 5.3–5.5 ppm (O'Connor et al., 1979).

The long-range coupling of the phosphorus nucleus with H2 is the third piece of evidence for an axial anomeric phosphate group, since only with that configuration can the molecule adopt the W conformation required for four-bond coupling to be observed (Hall & Malcolm, 1972).

Phosphorus–proton coupling constants can give information about the average rotational geometry of the phosphate group (O'Connor et al., 1979). Rotamer I in the notation of these authors has the phosphorus atom trans to the anomeric proton, and it is therefore beneath the sugar ring. In rotamer II the phosphate is gauche to H1 but adjacent to the N-acylated amino group, and in rotamer III it is adjacent to the ring oxygen atom. With the values for J_t and J_g adopted by O'Connor et al., the observed $J_{P,H1}$ value suggests that the phosphate spends 26% of its time trans to H1. α-D-Glucose 1-phosphate in solution behaves identically (O'Connor et al., 1979). No phosphorus–carbon coupling constants are available to determine the separate contributions from the other rotamers. Rotamer III, however, appears to give the largest contribution four-bond coupling, $J_{P,H2}$, in pyranose phosphates. Unfortunately, the magnitude of the splitting for each rotamer is not known from the model compounds that have been studied (Hall & Malcolm, 1972). Nevertheless, the four-bond coupling is smaller at pH 7.85 than at pH 5.5, indicating that as the phosphate becomes doubly ionized, it avoids the ring oxygen.

Anomeric Configuration of Aminoarabinosyl Phosphodiester. The number of possible conformations of the aminoarabinose ring is greater than that for the glucosamine. α-L-Aminoarabinose in the pyranose form is expected on energetic grounds to adopt the $^4C_1$ conformation in solution. The enantiomeric α-D-arabinose does exist as the corresponding $^1C_4$ form (Lemieux & Stevens, 1966) in order to make its three hydroxyl groups equatorial. α-L-Aminoarabinose should therefore have a diaxial H1–H2 coupling constant of about 8 Hz.

β-L-Aminoarabinose may be conformationally unstable, since there are two axial and two equatorial substituents in both pyranose ring forms. In either of these forms the H1–H2 coupling will be axial–equatorial, but only the $^4C_1$ conformer will have the anomeric proton equatorial.

The observed H1–H2 coupling constant of 3.5 Hz shows clearly that the β configuration is present, and the chemical shift of H1 shows that the anomeric proton is equatorial. An intermediate value for the chemical shift might have indicated conformational instability, but the observed shift of 5.57 ppm lies at the downfield end of the normal range for equatorial protons (O'Connor et al., 1979).

Acetylation of the amino group was performed in an attempt to determine the axial or equatorial nature of the amino group. Lichtenthaler & Emig (1968) have shown that axial *N*-acetyl groups have chemical shifts in the range 2.02–2.08 ppm, while equatorial groups have shifts from 1.90 to 1.95 ppm. As most of these data were obtained in chloroform solution and the reference signal used for the few aqueous samples is not clearly indicated, it is difficult to compare our result with these values. When the chemical shift of the *N*-acetyl group of de-*O*-acyl-LPS is referred to trimethylsilane in chloroform and the diamagnetic susceptibility correction appropriate for superconducting magnet geometry is made, its value is 1.96 ppm. The chemical shift of the equatorial *N*-acetyl group of 4-acetamido-4,6-dideoxy-D-mannose, also in D_2O, is 1.95 ppm (J. W. Redmond, unpublished results). This suggests that the aminoarabinose ring is undergoing interconversion between $^4C_1$ and $B_{1,4}$ forms with possible contributions from related skew forms, but spectra of more model compounds are required for confirmation.

The rotational behavor of the 4′-phosphates is also reflected in their phosphorus–proton coupling constants. The pH dependence of the population of the gauche conformer for the monester (Table II) confirms the suggestion that it is ionic interaction with the octulosonic acid residues that constrains the motion of the phosphate (Rosner et al., 1979a,b,c). The motion of the singly charged phosphate approaches free rotation, but the dianion avoids the acidic sugars. The difference between the splitting observed at pH 9 and that reported previously (Rosner et al., 1979a,b,c) is probably due to difficulties experienced by the previous workers in resolving the doublet.

The 4′ diester also has considerable rotational freedom, despite the presence of the aminoarabinose. The 6.8-Hz coupling between the phosphorus and H4′ leads to an estimate of 23% for the time spent in the conformer with the phosphorus trans to H4′. Even though the phosphate is equatorial to the glucosamine ring, considerable steric interactions will occur in this rotamer between the diester and axial atoms on the glucosamine ring. The time spent in this conformer is therefore quite high.

The anomeric proton of aminoarabinose has a slightly smaller coupling to the phosphorus. If the same parameters are applicable, this indicates a smaller value of 21% for the population of the trans rotamer compared to that of the other side of the diester. If one considers the number of conformations likely to be excluded because of steric crowding (Formanek & Weidner, 1981), the motion is remarkably free. It is unfortunate that lack of resolution will probably prevent similar information being obtained from the more biologically interesting case of the LPS molecule itself.

In summary, we have determined the anomeric configurations of two sterically important sites in the LPS molecule. The results for the reducing terminal glucosamine are as expected by some workers (Mühlradt et al., 1977) but not by others (Formanek & Weidner, 1981). Fortunately, the synthetic work has been carried out with material on the correct anomeric configuration (Inaga et al., 1981; Nashed & Anderson, 1981).

Acknowledgments

We are grateful to Dr. B. L. Reynolds, University of Adelaide, for providing *Salmonella minnesota* strain R595 and to Dr. P. Reeves, University of Aldelaide, for useful discussions. We thank Brigid Roberts for valuable technical assistance.

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Influence of the Mercury Blocking Reagent 2-Mercaptoethanol on the Spectroscopic Properties of Complexes Formed between Lysyltryptophyllysine and Mercurated Poly(uridylic acid)†

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ABSTRACT: Optical detection of magnetic resonance (ODMR) studies are reported for complexes formed between the tripeptide Lys-Trp-Lys and poly(5-HgU), both in the absence and in the presence of the blocking reagent 2-mercaptoethanol (ME). Complexes formed both with and without ME show characteristics of a heavy atom effect: quenching of Trp fluorescence, enhancement of the Trp phosphorescence quantum yield, a drastic reduction of the Trp phosphorescence lifetime, and the appearance of a Trp $|D| + |E|$ ODMR signal at ca. 4.2 GHz. Significant differences are found, however, in the photophysical properties of the complexes formed with and without ME. In the absence of ME, the Trp phosphorescence bands are broad, the 0,0 band is shifted to 415.3 nm, and the $|D| - |E|$ and $2|E|$ ODMR transitions are broad and poorly resolved. These features are characteristic of an inhomogeneous Trp environment. In the presence of ME, the phosphorescence peaks are narrower, with the 0,0 band shifted to 411.6 nm. The $|D| - |E|$ and $2|E|$ ODMR transitions are well resolved and shifted in frequency relative to the unblocked complex. These features point to a more homogeneous Trp environment in the presence of ME. UV difference spectra show hypochromicity in the poly(5-HgU) absorption band (indicating induced stacking) which occurs on binding of Lys-Trp-Lys with ME present, while in the absence of ME, hypochromicity occurs primarily in the Trp absorption bands. Reversal of these effects with added $NaClO_4$ occurs in both cases, but higher ionic strength is required with ME present. These results are consistent with the formation of stacked complexes in the presence of ME, but with additional types of complexes in its absence. The additional complexes formed in the absence of ME do not contribute to stacking of poly(5-HgU) and may involve direct binding of mercury to amines of Lys-Trp-Lys; binding occurs between Lys-Trp-Lys and the monomer 5-HgUTP in the absence of ME, but not when the Hg is blocked with ME.

Protein–nucleic acid interactions are by any measure among the most important processes operating in life. Numerous model systems have been subjected to extensive study in order to arrive at an understanding of the nature and the specificity of the interactions involved in protein–nucleic acid associations. In particular, studies of the complexes formed between oligopeptides and polynucleotides have provided some valuable information about these interactions (Hélène, 1981). Among the various types of interactions which may be operating in protein–nucleic acid associations, stacking interactions between aromatic amino acid residues and the nucleotide bases have been proposed as important in the specific recognition of nucleic acids by proteins (Gabbay et al., 1976; Toulmé & Hélène, 1977).

In this paper, we report on our investigations of complexes formed between the tripeptide Lys-Trp-Lys and the heavy atom derivatized polynucleotide poly(5-HgU). We make particular use of optical detection of triplet state magnetic resonance (ODMR)[1] in this work. ODMR spectroscopy has been developed in several laboratories over the last decade and has proven to be a useful method for studying the excited triplet state properties of proteins and nucleic acids (Maki & Zuclich, 1975; Kwiram, 1982; Maki, 1982). The external

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[1] Abbreviations: ODMR, optical detection of (triplet state) magnetic resonance; ME, 2-mercaptoethanol; ZFS, zero-field splitting; D and E, ZFS parameters; EDTA, ethylenediaminetetraacetic acid; EG, ethylene glycol; EGB, 50% v/v mixture of ethylene glycol and aqueous buffer; poly(U), poly(uridylic acid).

0006-2960/82/0421-6586$01.25/0