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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE ELABORATED BY *Haemophilus influenzae* TYPE d

PAULA BRANEFORS-HELANDER,
Institute of Medical Microbiology, University of Göteborg, S-413 46 Göteborg (Sweden)
LENNART KENNE, BENGT LINDBERG, KURT PETERSSON, AND PER UNGER
Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm (Sweden)

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ABSTRACT

The structure of the capsular polysaccharide elaborated by *Haemophilus influenzae* type d has been investigated, methylation analysis and n.m.r. spectrometry being the principal methods used. It is concluded that the polysaccharide is composed of repeating units having the structure: →4)-β-D-Glc*p*NAc-(1→3)-β-D-Man*p*NAcA-(1→. In addition, single residues of L-alanine, L-serine, or L-threonine, in the proportions 2:2:1, are linked, through their amino groups, to C-6 of the 2-acetamido-2-deoxy-β-D-mannopyranosyluronic acid residues. The degree of substitution (75–85%) varies for different preparations.

INTRODUCTION

The capsular antigens produced by *Haemophilus influenzae* types a, b, c, and f are of the teichoic acid type, whereas those from types d and e are polysaccharides. The structures of two polysaccharides elaborated by different strains of *H. influenzae* type e have recently been determined[1,2]. Preliminary studies[3] of the type d polysaccharide indicated that it was composed of 2-amino-2-deoxy-D-glucose and a 2-amino-2-deoxyhexuronic acid in the ratio 2:1. The uronic acid was tentatively identified as 2-amino-2-deoxy-D-glucuronic acid. The negative $[\alpha]_D$ value (−62°) further indicated that the sugar residues are β-D-linked. We now report further studies of this polysaccharide.

RESULTS AND DISCUSSION

The polysaccharide was isolated from a culture of *H. influenzae* type d, strain 868, by precipitation with cetyltrimethylammonium bromide, followed by chromatography on DEAE-Sepharose. The antigen had $[\alpha]_{578}$ −52°, and sugar analysis gave 2-amino-2-deoxy-D-glucose and, when preceded by treatment with alkali and subsequent carboxyl-reduction[4], also a comparable amount of 2-amino-2-deoxy-D-

mannose. The D configuration of the sugars was demonstrated by the method of Gerwig *et al.*[5].

The ^{1}H-n.m.r. spectrum showed, *inter alia*, signals for *N*-acetyl groups at δ 2.03 and 2.07 (6 H together). In the anomeric region, signals at δ 4.62 (1 H, very broad), 4.69 (1 H, broad), 4.84 (0.15 H, *J* not resolved), and 4.93 (0.85 H, *J* not resolved) were obtained. In addition, the spectrum showed signals for methyl groups at δ 1.20 (d, 0.5 H, *J* 6 Hz) and 1.37 (d, 1.0 H, *J* 7 Hz). These signals proved to be due to L-threonine and L-alanine covalently linked to the polymer. Amino acid analysis revealed that the polysaccharide also contained L-serine residues in a molar proportion approximately equivalent to that of L-alanine. The amino acids were proved to have the L configuration by g.l.c. of their acetylated (+)-2-butyl esters[6]. The polysaccharide thus contains L-alanine, L-serine, and L-threonine in the relative proportions 2:2:1. Approximately 0.85 mol of amino acid was found per 2 mol of sugar. Other samples of the polysaccharide contained smaller proportions of amino acid (~0.75 mol per 2 mol of sugar).

The amino acid linkages could be partly hydrolysed with 2M sodium hydroxide at 20° for 4 h. Under these conditions, alanine (30%), serine (85%), and threonine (50%) were released, as shown by amino acid analysis of the resulting polymer. The fraction of low molecular weight contained monomeric amino acids, but no peptides. This facile hydrolysis of amide linkages suggests neighbouring-group assistance. Essentially the same figures for alanine (34%) and threonine (52%) were obtained from the ^{1}H-n.m.r. spectrum. Virtually no *N*-acetyl linkages were hydrolysed, as shown by the ^{1}H-n.m.r. spectrum. This contained, *inter alia*, signals in the anomeric region at δ 4.64 (2 H, broad), 4.84 (0.67 H, *J* not resolved), and 4.92 (0.33 H, *J* not resolved). Comparison with the spectrum of the native material shows that two of the signals (δ 4.92 and 4.69) are shifted upfield (to δ 4.84 and 4.64, respectively) when the amino acids are removed. The results, in conjunction with evidence discussed below, indicate that ~33% of the uronic acid residues are substituted in the alkali-treated material. The corresponding figure for the native material is 85%.

On further alkaline treatment, almost all of the amino acids were released, but some degradation of the polysaccharide occurred as indicated by gel filtration of the product. This treatment was done before the carboxyl reduction, in order to get better yields of the *manno* derivatives in the sugar and methylation analyses.

Methylation analysis of the base-treated and carboxyl-reduced ($NaBD_4$) polysaccharide yielded comparable amounts of 2-deoxy-3,6-di-*O*-methyl-2-methylacetamido-D-glucose and 2-deoxy-4,6-di-*O*-methyl-2-methylacetamido-D-mannose-6-d_2. The combined evidence from the sugar and methylation analyses therefore indicates that the polysaccharide is composed of disaccharide repeating-units having the structure **1**.

→4)-β-D-Glc*p*NAc-(1→3)-β-D-Man*p*NAcA-(1→

1

The ^{13}C-n.m.r. spectrum of the base-treated polysaccharide (Table I) was

TABLE I

SIGNALS IN THE ^{13}C-N.M.R. SPECTRA OF THE CAPSULAR ANTIGEN FROM *H. influenzae* TYPE d AND RELEVANT REFERENCE SUBSTANCES

Substance		*Component*															
		2-Acetamido-2-deoxy-D-glucose						*2-Acetamido-2-deoxy-D-mannuronic acid*						N-*Acetyls*			
		C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C = O*		*-CH₃*	
H.i. type d antigen	pD 2	101.0	57.1	74.0	78.1	76.2	62.3	99.5	51.4	80.5	68.5	77.1[a]	—[b]	176.2		23.9	24.2
H.i. type d antigen	pD 8	101.4	57.3	74.3	78.3	76.5	62.3	99.7	51.4	80.9	68.7	77.1[a]	—[b]	176.3	176.3	23.9	24.2
H.i. type d, base-treated	pD 2	101.0	56.9	74.1	78.1	76.2	62.0	99.4	51.3	80.8	68.8	76.2	174.5	175.9	176.0	23.8	24.1
H.i. type d, base-treated	pD 8	100.9	56.9	74.2	78.4	76.2	62.1	99.3	51.3	80.8	69.0	78.4	176.5	175.9	175.9	23.8	24.1
H.i. type e, strain 8455	pD 2	102.5	56.2	84.4	70.4	77.3	62.5	101.3	53.9	72.1	79.3	77.3	173.6	175.9	176.5	23.8	24.1
H.i. type e, strain 8455	pD 8	102.1	55.9	84.6	70.5	77.4	62.4	101.4	54.4	72.2	79.5	78.8	176.0	176.0	176.5	23.8	24.1
Me β-D-Glc*p*NAc		103.2	56.7	75.2	71.2	77.1	62.0							176.1		23.3	
β-D-Man*p*NAcA[1]								94.3	54.9	72.8	69.3	76.2	—	176.8		23.2	

[a]The value is given for C-5 of the uronamide residue. Corresponding values for C-5 of the unsubstituted uronic acid are δ 76.2 (pD 2) and 78.3 (pD 8).
[b]Depends upon the substituent; see Table II.

TABLE II

^{13}C-N.M.R. SIGNALS GIVEN BY THE AMINO ACID RESIDUES AND ADJACENT CARBON ATOMS OF THE *H. influenzae* TYPE d ANTIGEN AND BY RELEVANT REFERENCE SUBSTANCES

Substance		*Component*																			
		L-*Alanine*			L-*Serine*			L-*Threonine*				D-*Glucuronic acid*							D-*ManNAcA*			
		C-1	*C-2*	*C-3*	*C-1*	*C-2*	*C-3*	*C-1*	*C-2*	*C-3*	*C-4*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*OMe*	*C-5*		*C-6*	
H.i. type d[a]	pD 2			18.1	174.6			174.6	59.8		20.8									171.9	171.9	171.5
H.i. type d[a]	pD 8	181.1	52.9	19.4	177.6	58.9	64.1	177.6	62.0	69.8	21.0								77.1	171.9	171.8	171.2
Me β-D-Glc*p*A-6(*N*)-L-Ala	pD 2	177.4	50.2	18.0								105.1	74.4	77.2	73.1	76.5	171.7	59.0				
Me β-D-Glc*p*A-6(*N*)-L-Ala	pD 8	180.9	52.6	19.4								105.0	74.5	77.2	73.3	76.6	171.2	59.0				
Me β-D-Glc*p*A-6(*N*)-L-Ser	pD 2				174.7	56.4	62.9					104.9	74.4	77.2	73.2	76.4	172.1	59.0				
Me β-D-Glc*p*A-6(*N*)-L-Ser	pD 8				177.3	58.5	63.8					104.8	74.5	77.2	73.3	76.6	171.8	58.9				
Me β-D-Glc*p*A-6(*N*)-L-Thr	pD 2							174.7	59.4	68.8	20.6	105.0	74.4	77.3	73.2	76.4	172.4	58.9				
Me β-D-Glc*p*A-6(*N*)-L-Thr	pD 8							177.7	61.7	69.4	20.9	104.9	74.5	77.2	73.4	76.5	172.0	58.9				
Me β-D-Glc*p*A	pD 2											105.0	74.5	77.2	73.1	76.6	174.7	58.9				
Me β-D-Glc*p*A	pD 8											105.0	74.8	77.5	73.6	77.6	177.2	58.9				

[a]Several of the expected chemical-shifts for the amino acid residues overlapped with signals from the polysaccharide backbone.

relatively simple, and tentative assignments of the signals, in good agreement with the suggested disaccharide repeating-unit, could be made by comparison with the capsular polysaccharide[1] of *H. influenzae* type e, strain 8455, and with reference substances of low molecular weight. Some signals were pD-dependent, the most pronounced differences being observed for the carboxylic acid carbon (δ 174.5 at pD 2; 176.5 at pD 8) and C-5 (δ 76.2 at pD 2; 78.4 at pD 8) of the 2-acetamido-2-deoxy-β-D-mannopyranosyluronic acid residue. As the chemical shift of the anomeric carbon is dependent on the aglycon[7], the $^1J_{C,H}$ coupling constants were measured for confirmation of the anomeric configuration[8]. The values obtained [$^1J_{C,H}$ 162 (δ 100.9) and 159 (δ 99.3)] accord with the β-D configuration of both residues. Irradiation of the "anomeric region" in the ^{1}H-n.m.r. spectrum, while measuring the ^{13}C nuclei, gave three decoupled signals [for the two anomeric carbons and one of the acetamido-substituted carbons (δ 51.3)]. This observation shows that one of the ^{1}H-n.m.r. signals in the "anomeric region" is derived from H-2 of the 2-acetamido-2-deoxy-β-D-mannopyranosyluronic acid residue (H-2 of 2-acetamido-2-deoxy-β-D-mannose gives a signal at δ 4.42).

The native polysaccharide, which contains amino acids, gives a more complex ^{13}C-n.m.r. spectrum. By comparison with the methyl β-D-glucopyranosiduronamides of L-alanine, L-serine, and L-threonine, respectively, assignments of the non-stoichiometric signals could be made (Table II). Of the signals given by the uronic acid residues, only those of C-5 and C-6 shifted significantly on going from acid to amide. These upfield shifts are also observed when comparing the spectra from the native and base-treated polysaccharide. No other signals have significant differences in chemical shift. This observation also verifies that the amino acids are linked as amides to the 2-acetamido-2-deoxy-β-D-mannopyranosyluronic acid residue.

The absence of other, non-stoichiometric signals in the ^{13}C-n.m.r. spectrum, the relative intensities of pertinent signals in the ^{1}H-n.m.r. spectrum, and the fact that no peptides were obtained on base treatment of the polysaccharide indicate that the amino acids are linked as single residues to position 6 of the uronic acid. On change of the pD from 8 to 2, several signals in the ^{13}C-n.m.r. spectrum were shifted. All signals from the amino acid residues were affected, as well as those from C-5 and C-6 of the unsubstituted uronic acid residue.

Several bacterial polysaccharides contain amino acid residues. Thus, lysine, amide-linked to D-galacturonic acid, has been observed in lipopolysaccharides from *Proteus mirabilis*[9]. In a cell-wall antigen from *Staphylococcus aureus*, the amino group of a 2-amino-2-deoxy-β-D-glucopyranosyluronic acid residue is acylated with *N*-acetyl-L-alanine[10]. In the bacterial polysaccharides earlier studied, only a single amino acid substituent has been observed, whereas in the capsular polysaccharide of *H. influenzae* type d, three different amino acids are present in non-stoichiometric amounts. This polysaccharide has also been investigated by Egan *et al.*[11], who have arrived at the same structure.

EXPERIMENTAL

General methods were the same as previously reported[1]. ^{13}C-N.m.r. spectra, without proton-decoupling, were obtained by using a gated decoupling technique. The selectively decoupled, ^{13}C-n.m.r. spectrum was obtained by irradiation at δ 4.75 in the ^{1}H-n.m.r. spectrum. The irradiation was strong enough to affect all protons in the region δ 4.5–5.0. In order to obtain better resolution in the anomeric region, some ^{1}H-n.m.r. spectra were recorded with a 200-MHz instrument (Bruker WP-200).

Investigation of the antigen. — (*a*) *Preparation. H. influenzae* type d, strain 868, was isolated at the Department of Clinical Microbiology, University of Göteborg, from an ear secretion. The capsular antigen, isolated and purified as previously described[1], had $[\alpha]_{578}^{20}$ −52° (*c* 0.6, water) and contained 0.1% of phosphorus (determined as described by Chen *et al.*[12]).

(*b*) *Alkaline treatment.* A solution of the antigen (30 mg) in 2M aqueous sodium hydroxide was kept at 20° for 4 h, and the polymer recovered by neutralisation, gel filtration, and freeze-drying; yield, 20 mg of polymer. The fraction of low molecular weight was analysed by paper chromatography. Only monomeric amino acids were observed.

(*c*) *Carboxyl reduction.* The base-treated antigen was treated as previously described[1]. Sugar analysis of the product yielded 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-mannose in the ratio 1:0.8.

(*d*) *Characterisation of the sugar and amino acid components.* Hydrolysis of the base-treated antigen, analysis of the sugar components, and determination of their absolute configurations were performed as previously described[1].

For qualitative and quantitative analysis of amino acids, the antigen was hydrolysed in 6M hydrochloric acid at 110° for 24 h, and the hydrolysate was analysed by using a Beckman 121-M amino acid autoanalyser.

For determination of the absolute configurations of the amino acids, a hydrolysate of the antigen (2 mg) was *N*-acetylated, and the dry product suspended in M (+)-2-butanolic hydrogen chloride. The suspension was kept at 100° for 25 h and then concentrated, and the residue was acetylated and analysed by g.l.c.–m.s. on an SP-1000 glass-capillary column, using a temperature programme, 150 → 200°. Comparison was made with samples prepared from authentic L-amino acids and *rac*- and (+)-2-butanol, respectively.

(*e*) *Methylation analyses.* These analyses were performed, as previously described[1,13,14], on the base-treated polysaccharide after carboxyl-reduction with sodium borodeuteride. The methyl ethers derived from the 2-amino-2-deoxy-D-mannuronic acid residues were thus readily distinguished from those derived from the 2-amino-2-deoxy-D-glucose residues.

Synthesis of methyl β-D-glucopyranosiduronamides of amino acids. — A solution of potassium (methyl β-D-glucopyranosid)uronate (100 mg, 0.41 mmol) and the methyl ester of the amino acid hydrochloride (2 mmol; L-alanine, L-serine, or L-threonine) in water (2.5 ml) was adjusted to pH 4.75. 1-Ethyl-3-(3-dimethylaminopropyl)carbo-

diimide hydrochloride (400 mg) in water (1.5 ml) was added. The solution was kept at 20° for 4 h, and, during the first hour, the pH was maintained at 4.75 by addition of 0.1M hydrochloric acid. The solution was passed through a column (20 × 1 cm) of Dowex 50(H^+) resin, which was washed with water (70 ml). The eluate was freeze-dried and treated with 0.1M sodium hydroxide (1 ml) for 20 min at 20°. Treatment with Dowex 50(H^+) resin, followed by freeze-drying, yielded the title compounds. The yields of the three amides were comparable (~50%). For n.m.r. analysis, the products were dissolved in deuterium oxide and the pD was adjusted by titration with sodium deuteroxide (0.1M) in deuterium oxide. In the ^{1}H-n.m.r. spectra, the anomeric protons gave signals at δ 4.45–4.47 (J 7.5 Hz) for the amides; *cf.* δ 4.36 (J 7.5 Hz) for the uronate. The CH_3 groups of the L-alanine and L-threonine residues gave signals at δ 1.38 (J 7.5 Hz) and 1.17 (J 6.5 Hz), respectively. The CH_2OD group of serine gave a signal at δ 3.88 (J 5.0 Hz).

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