Identification of phosphorylated 3-deoxy-manno-octulosonic acid as a component of *Haemophilus influenzae* lipopolysaccharide

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A phosphorylated 3-deoxy-manno-octulosonic acid (KDO) was released from the lipopolysaccharide (LPS) of the deep rough mutant (Rb⁺I69) of *Haemophilus influenzae* by acid hydrolysis. Both phosphorylated and dephosphorylated KDO, produced by treatment with alkaline phosphatase, were identified by gas chromatography-mass spectrometry after trimethylsilylation. This technique provides a rapid and reliable method for the identification of phosphorylated KDO in LPS.

INTRODUCTION

With possible rare exceptions such as *Shewanella putrefaciens* (Wilkinson *et al.*, 1973) and *Bacteroides* species (Hofstad, 1974), KDO is a universal component of bacterial LPS. It is located in the inner-core region and links the polysaccharide to the non-reducing glucosamine residue of lipid A through a relatively acid-labile α -(2→6)-keto linkage.

Determination of KDO in LPS is traditionally based on the thiobarbituric acid assay. However, when this assay was applied to *Haemophilus influenzae* LPS only a trace amount of KDO was revealed (Fletcher & Insel, 1978; Zamze & Moxon, 1987), suggesting that the KDO region of *H. influenzae* LPS is markedly different to that of most other bacterial LPSs. Larger amounts of KDO have been detected in *H. influenzae* LPS with the thiobarbituric acid assay by increasing the strength of acid hydrolysis of the LPS (Parr & Bryan, 1984; Zamze & Moxon, 1987) according to the method of Brade *et al.* (1983). This could be due to the release from the KDO of 4- and/or 5-linked substituent groups that would otherwise prevent its reaction with thiobarbituric acid. Inzana *et al.* (1985) suggested that KDO was present in *H. influenzae* LPS on the basis of the semicarbazide assay and the tentative g.c.-m.s. identification of a possible KDO acid-degradation product.

In the present paper we describe the unambiguous identification of KDO as a component of the LPS from the deep rough LPS mutant of *H. influenzae* (strain Rb⁺I69; Zwahlen *et al.*, 1985) by g.c.-m.s. analysis of its trimethylsilyl derivative after enzymic dephosphorylation. LPS from strain Rb⁺I69 was chosen for this study because it is deficient in all sugars terminal to the KDO region (S. E. Zamze & E. R. Moxon, unpublished work).

MATERIALS AND METHODS

Haemophilus influenzae strain Rb⁺I69 was provided by Dr. A. Zwahlen and strain Eagan (possessing a wild-type

LPS) is a clinical isolate (Anderson *et al.*, 1972). Ammonium 2-keto-3-deoxyoctonate, alkaline phosphatase (type VII-NL from bovine intestine) and *Salmonella minnesota* R595 (Re) LPS were obtained from Sigma Chemical Co. *N*-Acetyethanolamine phosphate was prepared from standard ethanolamine phosphate (Sigma Chemical Co.) by *N*-acetylation with acetic anhydride in saturated NaHCO₃.

Bacteria were grown at 37 °C with shaking in 800 ml of brain heart infusion broth (Oxoid) supplemented with 2 µg of haemin (Sigma Chemical Co.)/ml and 2 µg of NAD⁺ (Sigma Chemical Co.)/ml. Cells were harvested after 16 h (at stationary phase) and washed twice with 0.15 M-NaCl/0.015 M-sodium phosphate buffer, pH 7.2. LPS was extracted from strain Eagan with hot aqueous 45% (w/v) phenol (Westphal & Jann, 1965) and from strain Rb⁺I69 with aqueous 90% (w/v) phenol/chloroform/light petroleum (b.p. 40–60 °C) (2:5:8, by vol.) according to the procedure of Galanos *et al.* (1969). The phenol/chloroform/light-petroleum extraction was carried out on the freeze-dried cell material after phenol/water extraction. Both LPS samples were purified as previously described (Smith *et al.*, 1985).

KDO monosaccharide was released from the LPS (0.25–1 mg) by hydrolysis with 2 M-HCl for 20 min at 100 °C. The lipid A precipitate was removed by extraction three times with diethyl ether, and HCl was removed by rotary evaporation at 30 °C.

Samples were dephosphorylated by treatment with alkaline phosphatase (2 units) for 18 h at 37 °C in 0.2 ml of 0.2 M-NaHCO₃. Cations were removed from samples before trimethylsilylation by passage through a tandem column of Chelex X-100 (Na⁺ form) over Dowex AG-50 (H⁺ form), in water. For analyses in which detection of amino compounds was required, samples immediately after dephosphorylation were *N*-acetylated with acetic anhydride in saturated NaHCO₃.

Silylation was carried out with Sigma-sil A (15–40 µl) at room temperature for 18 h. The amount of KDO present per mg of LPS was determined by repeating the

Abbreviations used: KDO, 3-deoxy-manno-octulosonic acid (2-keto-3-deoxyoctonic acid); LPS, lipopolysaccharide; g.c.-m.s., gas chromatography-mass spectrometry.

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Fig. 1. Flame-ionization-detection chromatograms of the trimethylsilyl derivatives of *N*-acetylated and desalted hydrolysates of strain Rb⁺I69 LPS (a) before and (b) after treatment with alkaline phosphatase

The phosphorylated components P-1–P-6 (a) give rise to components K-1–K-4 (b) after enzymic dephosphorylation of the hydrolysate. The profile (retention times and relative peak areas) of components K-2–K-4 is identical with that given by the trimethylsilyl derivatives of the authentic KDO mixed α - and β -pyranose and -furanose anomers. The EtnAc-P, Gro-1-P, Gro-2-P and Glc-1 and Glc-2 peaks all have retention times and mass spectra identical with those of the trimethylsilyl derivatives of authentic *N*-acylethanolamine phosphate, glycerol 1-phosphate, glycerol 2-phosphate and glucose respectively.

analysis in the presence of a xylitol internal standard. A correction factor for the hydrolytic destruction and relative response factor of KDO was obtained by treating authentic KDO in the presence of xylitol in a fashion identical with that used for the sample.

Combined g.c.–m.s. was performed on a Hewlett–Packard 5996 gas chromatograph–mass spectrometer equipped with a direct on-column injector. The trimethylsilyl derivatives were analysed on a bonded SE-30 fused-silica capillary column (20 m $\times$ 0.32 mm) with a temperature programme of 140 °C (2 min) to 250 °C (10 min) increased at 6 °C/min. He was the carrier at 3 ml/min. The g.c. eluate was split for simultaneous mass-spectral analysis for identification and flame ionization detection for quantification. Spectra were recorded using electron-impact ionization at 70 eV with an ion-source temperature of 150 °C and pressure of 2.7 mPa (2×10^{-5} Torr).

Glucosamine was liberated from lipid A by total

hydrolysis of LPS (1–2 mg) with 6.1 M-HCl at 105 °C for 4 h under N₂ (Smith *et al.*, 1985) and analysed by g.c. as the alditol acetate derivative with a Pye–Unicam 204 instrument equipped with a flame ionization detector. Inositol (200 nmol) was added as the internal standard. Authentic *N*-acetylglucosamine (200 nmol) was similarly treated to account for losses during hydrolysis. Alditol acetates were analysed on a packed column (0.9 m $\times$ 2 mm internal diam.) of 3% SP2340 on 100/120 Supelcoport with a temperature programme of 180 °C to 240 °C at 2 °C/min and N₂ as the carrier at 40 ml/min.

RESULTS AND DISCUSSION

G.c.–m.s. analysis of the trimethylsilyl derivatives of desalted strain Rb⁺I69 LPS acid hydrolysates revealed the presence of phosphorylated components (Fig. 1a; components P-1–P-6), which were readily identified by

Fig. 2. Mass spectra of the trimethylsilyl derivatives of dephosphorylated KDO from strain Rb⁺I69 LPS

(a) Mass spectrum of component K-1. The spectrum is identical with that of an acid-degradation product of authentic KDO.
 (b)–(d) Mass spectra of components K-2, K-3 and K-4 respectively. These spectra are identical with those of authentic KDO.

the prominence of the diagnostic ions at m/z 315 $[(\text{Me}_3\text{SiO})_3\text{POH}]^+$ and m/z 299

and had the expected retention times of monophosphorylated sugar trimethylsilyl derivatives. Removal of cations before formation of the derivatives was found to be a prerequisite for the successful g.c.–m.s. analysis of phosphorylated samples.

When the same LPS acid hydrolysate was treated with alkaline phosphatase, the components P-1–P-6 shown in Fig. 1(a) were no longer observed. The disappearance of

the phosphorylated material coincided with the appearance of four new components (Fig. 1b; components K-1–K-4). Components K-2–K-4 had retention times and mass spectra identical with those of the pyranose and furanose anomers of authentic KDO. The component K-1 is thought to be an acid-degradation product, as an identical product is found in acid hydrolysates of authentic KDO but is absent from non-hydrolysed KDO. The dephosphorylated KDO identified in strain Rb⁺I69 LPS was indistinguishable from 3-deoxy-manno-octulosonic acid released from *Salmonella* R595 LPS (results not shown). We have therefore assigned a *manno* configuration to the KDO in *H. influenzae* LPS. The KDO components (Fig. 1b, peaks K-2–K-4) are

Fig. 3. Mass spectra of the trimethylsilyl derivatives of phosphorylated KDO from strain Rb⁺I69 LPS

(a) Mass spectrum of component P-1, assigned to the phosphorylated form of the KDO acid-degradation product. (b) Mass spectrum of component P-4, assigned to a KDO monophosphate, and representative of the similar spectra found for components P-2–P-6.

characterized by ions at m/z 655 ($[M - \text{Me}]^+$) and/or m/z 553 ($[M - \text{CO}_2\text{Me}_3\text{Si}]^+$), m/z 463 ($[M - \text{CO}_2\text{Me}_3\text{Si} - \text{Me}_3\text{SiOH}]^+$), m/z 373 ($[M - \text{CO}_2\text{Me}_3\text{Si} - 2\text{Me}_3\text{SiOH}]^+$) and m/z 283 ($[M - \text{CO}_2\text{Me}_3\text{Si} - 3\text{Me}_3\text{SiOH}]^+$) (see Fig. 2).

Mass-spectral analysis (see Fig. 3a) suggests that the phosphorylated component P-1 (Fig. 1a) may be the phosphorylated version of the KDO acid-degradation product K-1 (Fig. 1b), as the former contains an m/z 645 ion that is replaced by an m/z 493 ion in the dephosphorylated component, a loss of 152 mass units consistent with the predicted replacement of $-\text{PO}_4(\text{Me}_3\text{Si})_2$ by $-\text{OMe}_3\text{Si}$. Each of the other five phosphorylated components P-2–P-6 (Fig. 1a) revealed a similar mass spectrum and they are all presumed to be KDO phosphate. The mass spectrum of the most abundant of these components, P-4, is shown in Fig. 3(b). The position of the phosphate group cannot be unambiguously determined from the mass spectrum, although the poor reactivity of KDO in the thiobarbituric acid assay would imply substitution at C-4 or C-5.

In addition to KDO, g.c.-m.s. analysis of *N*-acetylated hydrolysates of strain Rb⁺I69 LPS revealed the presence of both *N*-acetylglycerol phosphate and glycerol phosphate (Fig. 1a); the latter was detected as an equilibrium mixture of the 1- and 2-phosphates because of acid-catalysed phosphate migration. Whereas ethanolamine phosphate is a common LPS component (e.g. Lehmann *et al.*, 1971), the presence of glycerol phosphate in LPS is highly unusual. Both ethanolamine phosphate and glycerol phosphate could occur as pyrophosphate substituents of intact strain Rb⁺I69 LPS, either substituting the KDO (subsequently detected as KDO monophosphate in hydrolysates) or the lipid A glucosamine backbone, or both.

It is unlikely that glycerol phosphate came from phospholipid impurities, as g.c. analysis of fatty acids (results not shown) in strain Rb⁺I69 LPS revealed only *H. influenzae* lipid A components ($\text{C}_{14:0}$ and 3-hydroxy- $\text{C}_{14:0}$) and traces of $\text{C}_{16:0}$ and $\text{C}_{16:1}$ in a similar molar ratio to that found in strain Eagan LPS (Zamze & Moxon, 1987).

A small amount of glucose, approx. 0.1 mol/mol of KDO, of unknown origin, was also detected in strain Rb⁺I69 LPS (Figs. 1a and 1b). Quantitative analysis of strain Rb⁺I69 LPS, including correction for relative response factors and hydrolytic destruction, gave (means $\pm$ s.d.) 0.26 ± 0.03 μmol of KDO/mg of LPS and 0.48 ± 0.02 μmol of glucosamine (lipid A)/mg of LPS, implying a KDO/glucosamine molar ratio of 1:2. Assuming that lipid A is based on a disaccharide of glucosamine (e.g. Rietschel *et al.*, 1983), this molar ratio suggests that one KDO residue is present per LPS molecule. The ratios of ethanolamine phosphate and glycerol to KDO were approximately 0.6 and 0.3 respectively.

Enterobacterial LPS possesses a KDO trisaccharide (e.g. Lüderitz *et al.*, 1983). Investigations of the LPS from rough mutants of *Salmonella minnesota* (Brade *et al.*, 1985; Tacken *et al.*, 1986) and *Salmonella godesberg* (Brade *et al.*, 1984) have shown the structure of the trisaccharide to be linear, with one KDO residue in the main chain and an α -(2 $\rightarrow$ 4)-linked KDO disaccharide as a branch. Our analysis suggests that this structure is not present in *H. influenzae* LPS. Precedence for structural variation in the LPS KDO region exists for other families of bacteria. For example, LPS from species of *Rhodopseudomonas* (Strittmatter *et al.*, 1983), *Xanthomonas* (Volk *et al.*, 1972) and from members of the Vibrionaceae (Banoub *et al.*, 1983) all lack the branch

KDO disaccharide. *H. influenzae* LPS would appear to bear a close resemblance to *Vibrio cholerae* LPS, which also contains a single phosphorylated KDO residue (Brade, 1985). The latter identification was made by g.c.-m.s. analysis of reduced and permethylated phosphorylated KDO. In this case the phosphate could be assigned to the C-5 position of the KDO, although by this method the yields of the phosphorylated KDO derivatives were low.

KDO is extremely susceptible to acid degradation; however, the extent of destruction is the same for authentic KDO and for KDO liberated from LPS under a given hydrolysis condition (Brade *et al.*, 1983). It should therefore be possible to obtain a reasonably accurate estimation of the amount of KDO present in LPS. The procedure described, i.e. treatment of LPS hydrolysates with alkaline phosphatase followed by desalting and g.c.-m.s. analysis of the trimethylsilylated products, gives a rapid and reliable method for the identification of KDO in LPS as an alternative to the thiobarbituric acid assay and further benefits from being independent of KDO phosphorylation.

Although use of a deep rough LPS from *H. influenzae* helped to simplify this analysis, we were able similarly to make a clear identification of both phosphorylated and dephosphorylated KDO in LPS from the wild-type strain Eagan.

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