Mass spectrometric analysis of prenyl phosphates and their glycosylated forms*

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Three different mass spectrometric methods suitable for the analysis of polyprenyl and dolichyl phosphates and their glycosylated forms are described. Fast atom bombardment mass spectrometry (FAB MS) of glycosyl monophosphopoly sugars produces negative ions characteristic of the intact molecule. Tandem mass spectrometry of (M-H)\(^-\) anions allows the determination of masses of both glycosyl and lipid moieties. Thus, for example, FAB-MS/MS of a mixture of native glycosyl monophosphopoly sugars isolated from ethambutol-treated Mycobacterium smegmatis enabled us to detect two novel pentosyl monophosphopoly sugars.

Two other methods are proposed for the analysis of prenyl phosphates, as these compounds do not produce fragments in FAB-MS/MS at low collisional energy. By Desorption Electron Impact Ionization (DEI) an intense (M-H\(_2\)PO\(_4\))\(^-\) ion as well as fragments corresponding to the successive loss of isoprene residues (68 Da) can be observed. Alternatively, Desorption Chemical Ionization yields ions corresponding to the loss of 66, 78 and 98 Da (i.e., of a part or the entire phosphate moiety) of a prenyl phosphate molecule. Tandem mass spectrometry of the (M-H-98)\(^-\) ion gives a series of intense fragments differing by 58 mass units over the whole mass range.

Polytrenyl and dolichyl phosphates can be analyzed by standard electron impact method, but they give rather weak signals. To improve the sensitivity, samples have to be dephosphorylated by enzymatic and chemical methods [1–3]. Hydrolytic procedures have some drawbacks; the most important are degradation processes which may occur during hydrolysis. This is especially true in the case of α-unsaturated polyprenyl phosphates which, when submitted to acid hydrolysis, give a complicated mixture of alcohols and hydrocarbons [4].

More recently, Fast Atom Bombardment (FAB-MS) was applied for the analysis of prenyl phosphates [5]. Pseudomolecular negative ions and fragment ions were observed, and suitability of different matrices was tested.

For complex mixtures, however, the only useful information from a FAB-MS spectrum is the molecular mass. Tandem mass spectrometry is then needed to get structural data. Yet, pseudomolecular anions of monophosphorylated prenols do not produce fragments at 98 Da. Furthermore, no mass spectra of glycosylated forms of prenyl phosphates have been reported in the literature.

Mycobacteria represent a rare example of organisms which possess two structurally different prenyl phosphates. One is a C\(_{50}\)-decaprenyl phosphate (M\(_{\text{in}}\) 778 Da) [7] and the other is an

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even shorter, partially saturated C55-octahydroheptaprenyl phosphate (M_n 582 Da) [8]. For comparison, in most other bacteria, a C55-undecaprenol is regarded as a typical carrier lipid [1, 9–11], with the exception of archaeabacteria in which an α-saturated C60-dolichol rather, than a polyprenol, was found [12]. Both mycobacterial polyprenyl phosphates served as mannosyl acceptors from GDP-[14C]mannose in in vitro assays.

Only very recently, we have described a family of native glycosyl monophosphopolyprenels from Mycobacterium smegmatis and demonstrated the presence of polyprenyl-P-pentoses within this family [13–15]. In fact, the first hints of the existence of polyprenyl-P-pentoses in Mycobacterium smegmatis emerged from FAB-MS analysis. The search has been continued leading to the elucidation of the complete structure of one member of this family, i.e. decaprenyl-P-arabinose [15, 16]. It has been shown also that ethambutol, an antituberculosis drug known to inhibit the synthesis of a mycobacterial cell wall polysaccharide, arabinogalactan [17], provokes an accumulation of the lipid-linked arabinose. As far as we know, this is the first report of a polyprenyl-P-pentose in a living organism.

In this paper, we describe three different methods suitable for the analysis of polyprenyl and dolichyl phosphates as well as their glycosylated forms.

MATERIALS AND METHODS

**Bacteria.** Mycobacterium smegmatis, strain 1515 from Trudeau Mycobacterial Collection, was grown at 37°C on Nutrient Broth (Difco Laboratories, Detroit, MI, U.S.A.) with shaking. Ethambutol was added to the culture at mid-log phase, and growth was continued for another 5 h. Cells were harvested by centrifugation.

**Isolation of glycosyl monophosphopolyprenels** was performed as described by Wolucka [13]. Briefly, the purification procedure included:

1. Extraction of cells with ethanol followed by a partition of the stripped extract into a mixture of CHCl_3/CH_3OH/H_2O (8:4:3, by vol.);
2. DEAE-cellulose chromatography of the lipidic material;
3. Mild-alkaline hydrolysis of the DEAE-cellulose fractions containing mild-acid-labile monosaccharides; and finally,
4. Re-chromatography of the mild-alkaline stable lipids on a DEAE-cellulose column. The so obtained family of polyprenyl-P-sugars was analyzed by mass spectrometric methods. Mycobacterial decaprenyl phosphate was obtained by a spontaneous hydrolysis of the purified decaprenyl-P-arabinose, as described by Wolucka et al. [16].

**Analytical procedures.** Sugars were converted to the corresponding alditol acetates [18] and analyzed by GC on a Durabond 1, fused silica column (30 m) (J&W Scientific, Rancho Cordova, CA, U.S.A.) operating isothermically at 260°C.

FAB-MS/MS was performed on a Finnigan MAT TSQ 70 triple quadrupole Mass Spectrometer with an Ion Tech FAB gun operating at 7 keV and 1 mA with xenon. In MS/MS experiments, the collision offset voltage was 10 V and the pressure of target gas (xenon) was 0.7 mTorr. Samples were dissolved in a mixture of CHCl_3/CH_3OH (2:1, v/v) to a concentration of about 3 mg/ml. One microlitre of the solution was applied to a thioglycerol matrix.

In Desorption Electron Impact and Desorption Chemical Ionization experiments, 1 μl of a solution for analysis was first evaporated on a rhodium wire. The wire was then introduced into the ion source and heated either close to the electron beam (DEI) or in the chemical ionization plasma (DCI) composed of a mixture of CH_4 and N_2O (3:1, v/v).

Fragment ion spectra were obtained by focusing the selected ion with the first (MS1) mass spectrometer, fragmenting it by collision induced dissociation (CID) in the collision cell with xenon, and analyzing the fragments with the second mass spectrometer (MS2).

RESULTS AND DISCUSSION

The adapted procedure of purification allowed us to obtain from ethambutol-treated Mycobacterium smegmatis cells a family of glycosylated monophosphopolyprenels, as judged on the basis of their chemical and chro-
matographic characteristic, $^1$H-NMR spectrum and sugar analysis [15, 16]. The latter revealed the presence in this fraction of mainly two mild-acid-labile monosaccharides: arabinose and mannose.

Negative ion FAB and FAB-MS/MS of glycosylated monophosphopolyprenols from Mycobacterium smegmatis

A negative ion FAB spectrum of a mixture of glycosyl monophosphopolyprenols isolated from Mycobacterium smegmatis is shown in Fig. 1A. The most intense ion in the high mass region is observed at m/z 909. The signals at m/z 1017 and m/z 1125 are adducts of the m/z 909 with one and two molecules of the matrix (thioglycerol); $M_m = 108 \text{ Da}$, respectively. Tandem mass spectrometry of the m/z 909 ion gives information on its structure (Fig. 1B). Indeed, the product ions at m/z 891, 819 and 777 are present. The m/z 777 corresponds to the mass of (C33) decaprenyl phosphate, while the difference between 909 and 777, i.e. 132 Da, is the mass of pentosyl residue. The fragment at m/z 819 results from a cleavage within the sugar ring. Therefore, the m/z 909 ion represents a pseudomolecular ion of decaprenyl-P-pentose, more specifically: decaprenyl-P-arabinose.

Two other glycosylated derivatives have been identified in the same sample, at m/z 743 (Fig. 1C) and 713 (Fig. 1D). The CID spectrum of both ions displays an anion at m/z 581 which corresponds to octahydroheptaenyl phosphate. Thus, the ions at m/z 713 and 743 differ in their sugar moieties; a pentosyl residue (132 Da) is associated with the m/z 713 ion (581+132) and a hexosyl residue (162 Da) with the m/z 743 ion (581+162). This is confirmed further by the presence of a signal of hexosyl-PO$_3$ at m/z 241 in the MS/MS spectrum of the m/z 743 ion. Accordingly, a signal of pentosyl-PO$_3$ at m/z 211 is observed in the product ion spectrum of the 713 ion. Both ions, i.e. m/z 713 and 743, give in FAB-MS/MS a fragment ion at m/z 623 resulting from a fracture across the sugar ring.

![Fig. 1. Negative ion FAB-MS/MS analysis of glycosyl monophospho-polyprenols isolated from ethambutol-treated Mycobacterium smegmatis. (A) FAB-MS spectrum. (B) Fragment ion spectrum of m/z 909 ion (decaprenyl-P-arabinose). (C) Fragment ion spectrum of m/z 743 ion (octahydroheptaenyl-P-mannose). (D) Fragment ion spectrum of m/z 713 ion (octahydroheptaenyl-P-arabinose).](image-url)
(Fig. 1C and 1D) analogous to that observed in the case of decaprenyl-P-arabinose (m/z 909; Fig. 1B). Thus, on the basis of sugar analysis and FAB-MS/MS data, we conclude that the ions at m/z 713 and m/z 743 are pseudomolecular ions of arabinosyl- and mannosyl-monophosphochoctahydroheptaprenol, respectively.

The FAB-MS spectrum of the family of mycobacterial polypropenyl-P-sugars shows also the presence of ions at m/z 777 and 581; the former can be attributed to decaprenyl phosphate and the latter to the octahydroheptaprenyl phosphate. The ion at m/z 885 represents an adduct of the m/z 777 with one molecule of the thioglycerol matrix. As these ions give only little fragmentation by MS/MS, we describe here two other methods to analyze the phospholipid moieties.

Positive ion DEI of monophosphopolyproprenols

Desorption Electron Impact spectrum of the mycobacterial decaprenyl phosphate demonstrated an intense (M-H2PO4)+ ion (Fig. 2). Fragments corresponding to the successive loss of isoprene residues (68 Da) with the most intense ion at m/z 69 derived from the unsaturated ω-residue [19] were observed as well. This method of analysis of prenyl phosphates is much more sensitive than the direct insertion probe electron impact.

Negative ion DCI of monophosphopolyproprenols

Alternatively, Desorption Chemical Ionization (DCI) in negative ion mode can be applied for the analysis of prenyl phosphates (Fig. 3). The spectrum displays ions corresponding to the loss of 66, 78 and 98 Da, i.e. of a part of the entire phosphate moiety (Fig. 3A). The molecular ion is not observed. Product ions spectrum of the (M-H-98)+ parent gives a series of ions differing by 68 mass units accompanied by other fragments, 14 mass units larger or smaller (Fig. 3B). With a proper selection of the collision energy, very intense fragments are observed over the whole mass range. The mechanism of ion production by this method is not well understood; experiments with deuterium-labelled compounds would be very helpful for the elucidation of the underlying mechanism.

In summary, we have described here three sensitive methods for the analysis of prenyl phosphates and their derivatives. FAB-MS seems to be a method of choice to get data about molecular mass of these compounds. Moreover, FAB-MS/MS gives information about the nature of lipid as well as glycosyl parts of prenyl-P-sugars and, importantly, it can be applied in the case of mixtures. On the other hand, DEI and DCI can be useful to gather more details necessary for the elucidation of the lipid structure.

Altogether, these methods may bring new insights for our understanding of the glycosylation processes in general. We believe also that FAB-MS/MS will be very useful for the analysis of prenyl-pyrophosphoholigosaccharides, as diphosphates can be expected to give even better signals than the monophosphorylated species.

Fig. 2. Positive ion Desorption Electron Impact (DEI) spectrum of the mycobacterial decaprenyl phosphate.

Fig. 3. Negative ion Desorption Chemical Ionization analysis of the mycobacterial decaprenyl phosphate. (A) DCI spectrum; (B) MS/MS product ion spectrum of m/z 679 ion.
REFERENCES


