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Polysaccharide structural variability in mycobacteria: identification and characterization of phosphorylated mannan and arabinomannan

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Abstract Arabinomannan (AMannan) and mannan (Mannan) are major polysaccharides antigens of the mycobacterial capsule. They are highly related to the lipoarabinomannan (LAM) and lipomannan (LM) lipoglycans of the cell wall, known to participate to the immunopathogenesis of mycobacterial infections. Here we present the identification of two related polysaccharides from *Mycobacterium kansasii* that co-purified with AMannan and Mannan. Structural analysis using GC, MALDI-MS and NMR clearly established these molecules as non-acylated phosphorylated AMannan and Mannan designated P-AMannan and P-Mannan, respectively. These glycoconjugates represent a new source of polysaccharide structural variability in mycobacteria and constitute unique tools for structure-activity relationship studies in order to investigate the role of fatty acids in the biological functions of LAM and LM. The potential participation of these polysaccharides in influencing the outcome of the infection is also discussed.

Keywords *Mycobacterium kansasii* · Lipoarabinomannan · Lipomannan · Lipoglycan · NMR

Abbreviations

Ara	arabinose
ConA	concanavalin A
GC	gas-chromatography
HPAEC	high pH anion exchange chromatography
Ins	inositol
MALDI-TOF	matrix assisted lazer desorption ionisation coupled to time of flight analyser
Man	mannose
Man-ol	mannitol
Me-Glc	methyl glucoside
NMR	nuclear magnetic resonance
TFA	trifluoroacetic acid
TLC	thin layer chromatography

Introduction

Although the concept of a polysaccharide capsule has not been widely accepted, there is considerable evidence that supports the existence of a capsule surrounding the mycobacterial surface [1]. This outermost layer may correspond to the electron-transparent zone found immediately outside the mycobacterial cell wall [1]. It is composed mainly of polysaccharides, and there is a small amount of protein and almost no detectable lipid [1]. Lemassu and Daffe demonstrated that the major components of the *M. tuberculosis* capsule are polysaccharides such as arabinomannan (AMannan) and mannan (Mannan) [2]. There is considerable evidence that antibody to the *M. tuberculosis*

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polysaccharides can modify the course of infection to the benefit of the host [3]. A monoclonal antibody to mycobacterial AMannan was shown to prolong survival when it was incubated with *M. tuberculosis* before infection of mice [4], indicating that cell surface carbohydrates could include protective antigens. However, little is known regarding to the role that AMannan/Mannan play in the physiopathology of mycobacterial infection. There is one report suggesting that AMannan displays an immunosuppressive effect on the activation of human lymphocytes [5]. On the other hand, lipoarabinomannan (LAM) and lipomannan (LM), consisting of AMannan and Mannan bearing a mannosyl-phosphatidyl-*myo*-inositol anchor, are the major cell wall lipoglycans, and have been extensively studied [6]. Both LM and LAM play important functions in modulating the host immune response [6]. LAM is involved in the inhibition of phagosome maturation [7], apoptosis and interferon (IFN)-gamma signalling in macrophages and interleukin (IL)-12 cytokine secretion of dendritic cells [8], all being important processes for the host to mount an efficient immune response. In contrast to LAM, LM known to be a direct biosynthetic precursor of LAM, displays strong pro-inflammatory activities [9–11] and appears to be a potent matrix metalloproteinase-9 inducing factor [12].

We have recently determined the structure of LAM from *M. kansasii* and found that it belongs to the mannose-capped family of LAM, which appears to be a general feature of pathogenic mycobacterial species [6, 13]. *M. kansasii* is one of the most frequent atypical mycobacterial pathogens isolated from clinical specimens. Infection with *M. kansasii* can cause pulmonary disease similar to tuberculosis in patients with human immunodeficiency virus (HIV) infection or with pre-existing pulmonary disease [14, 15]. Since the start of the AIDS epidemic, a vast increase in *M. kansasii* infection incidence has been observed [16]. However, little is known about pathogenicity of *M. kansasii*, its mode of transmission, natural reservoir or virulence factors. Because mycobacterial surface carbohydrates have been proposed to influence adherence to macrophage receptors and phagocytosis of the bacteria, thus affecting the outcome of the infection [17], we have focused our attention in trying to identify and characterise novel LM/LAM-related carbohydrates that may be associated to the outermost portion of the *M. kansasii* envelope.

In this study, we have used a detergent phase separation method to allow to separate the amphipatic lipoglycans LM/LAM from their hydrophilic polysaccharides structurally related AMannan/Mannan. We describe the identification of new hydrophilic polysaccharides which co-purified with capsular AMannan/Mannan. Detailed structural analysis revealed that these carbonhydrates mainly consist of non-acylated phosphorylated AMannan/Mannan. Studies of these molecules should help to the design of structure-activity relationship studies and to establish a structural basis for the biological activities of LAM/LM.

Materials and methods

Strain and culture conditions

M. kansasii PHRI 901 was grown on Middelbrook 7H11 agar plates supplemented with 10% oleic acid/albumin/dextrose/catalase enrichment or under shaking in Sauton medium at 37°C.

Purification of neutral polysaccharides

Cells were harvested, washed in PBS (20 mM K_2HPO_4 pH 7.5, 0.15 M NaCl) and resuspended in lysis buffer (8% (v/v) Triton X-114 in PBS, 5 mM EDTA, 10 mM $MgCl_2$). Cells were then heat-inactivated, disrupted using a French Pressure Cell and stirred overnight at 4°C. Cellular debris and insoluble material were removed by centrifugation (27,000 g, 30 min, 4°C) and phase partition was induced at 37°C. Polymers were purified from the Triton-depleted upper phase according to Lemassu *et al*. [2]. This aqueous phase was collected and dialyzed against water for 3 days. Contaminating proteins were precipitated in 60% ammonium sulphate and the soluble fraction was dialyzed against water. Most glucans were extracted from this mixture by chloroform/methanol/water partition (3:4:3 v/v/v). Polymers were precipitated from aqueous phase overnight at 4°C with six volumes of ethanol. The precipitate was collected after centrifugation at 14, 000 g for 1 h, dissolved in water, dialyzed against water for 3 days and lyophilised. Material was dissolved in 10 mM Tris/HCl pH 8.0 and chromatographed on a DEAE-Trisacryl (IBF, France) equilibrated with the same buffer. Neutral faction was separated on a Bio-Gel P10 column eluted by 1% acetic acid in three fractions of different apparent molecular masses according to their monosaccharide contents (AMannan, Mannan and Me-Glc). AMannan- and Mannan-containing fractions were further purified following the same chromatography procedures in order to eliminate cross contaminations. In addition, contaminating glucose polymers were removed from Man-containing fractions by affinity chromatography on a ConA-Sepharose 4B (Pharmacia) column successively eluted with ConA buffer (5 mM sodium acetate pH 5.2 containing 0.1 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and 1 M Me-Glc in ConA buffer. AraMannan- and Mannan-containing fractions were finally separated in two different sub-fractions (AMannan1, AMannan2, Mannan1 and Mannan2) by

chromatography using Bio-Gel P10 and Bio-Gel P6 columns equilibrated and eluted in water.

LM was purified from *M. kansasii* as previously described [13]. Deacylation of LM was performed using NaOH 0.1 M in H_2O at 37°C for 2 h. Desalting was done by gel filtration chromatography on a BioGel P4 (BioRad) column eluted in water prior to NMR analysis.

Composition analyses

Monosaccharide and inositol composition was established by GC as trimethylsilyl derivatives before and after reduction of the intact polysaccharides [18]. Polysaccharides were reduced in a solution 20 mg/ml $NaBH_4$ in NH_4OH 0.05 M for 2 h. Solution was dried, repetitively codistilled in 10% acetic acid in anhydrous methanol (v/v), demineralised with cation exchanger DOWEX 50×2 (200–400 mesh, H+ form) and freeze-dried before methanolysis. Monosaccharide composition of glycoconjugates eluted from the different chromatography columns were systematically assessed online by TLC chromatography. An aliquot of each tube was hydrolysed in TFA 4N, 4 h at 100°C, run on silica gel 60 thin layer chromatography (Merck) in water-saturated phenol. Samples were detected following staining with orcinol reagent and charring.

Phosphate content was measured by HPAEC on an IonPac AS4A column (Dionex) as previously described for sulphate [19].

MALDI-MS

The molecular masses of polysaccharides were measured by MALDI-TOF on a Voyager Elite reflectron mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA), equipped with a 337 nm UV laser. Samples were prepared by mixing directly on the target 1 μL of water diluted polysaccharide solution and 1 μl of 2,5-dihydroxybenzoic acid matrix solution (10 mg/ml dissolved in Acetonitrile/H_2O).

NMR spectroscopy

Samples were exchanged two times with ^2H_2O (99.97% ^2H, euriso-top®, Saclay France) and finally dissolved in 250 μl of Me_2-SO-d_6 (DMSO) in Shigemi® (Allison Park, USA) tubes matched for DMSO. Both ^1H and ^{13}C chemical shifts (δ) were expressed in ppm downfield from the signal of methyl group of DMSO at δ 2.52 and 40.98 respectively and δ ^{31}P was expressed from external H_3PO_4 (85%) (δ_{31p} 0) at 343 K. The samples were analysed on a Bruker® Avance-400 spectrometer equipped with TXI probehead (^1H, ^{13}C and ^{31}P observed at 400.33, 100.66 and 162.5 MHz, respectively). The Bruker pulse programs were used and optimized (pulse lengths and delays) for each experiment.

Results and discussion

Identification and purification of novel hydrophilic polysaccharides in *M. kansasii*

Cells were resuspended in lysis buffer containing Triton X114 and disrupted using a French Pressure Cell. Lipoglycans were then separated from polysaccharides by phase separation at 37°C. This methodology enabled to recover high yields of LM/LAM as well as their non-acylated forms. Detailed structural analysis of LM and LAM from *M. kansasii* has been reported previously [13]. Gel exclusion chromatography was used to fractionate the various *M. kansasii* carbohydrates present in the detergent-depleted phase (hydrophilic phase) during the Triton X-114 phase separation method. After separation of total polysaccharides on a Bio-Gel P10, three major types of neutral glycoconjugates with different monosaccharide compositions were purified from *M. kansasii* which appear to be present in similar amounts (Fig. 1a). According to their compositions, the polysaccharides were tentatively identified as AMannans, Mannans and partially methylated Glucans, in accordance with previous studies on exocellular polysaccharides from *M. tuberculosis* [2, 20]. The three components were further separated on Bio-Gel P10 gel filtration under identical experimental conditions and Mannan was finally purified by ConA affinity chromatography. After complete purification, GC analysis revealed that AMannan, Mannan and partially methylated Glucan were composed of Ara/Man (1:1.2), Man and 6-*O*-methylGlc/Glc (1.3:1), respectively.

An additional purification step including gel filtration chromatography on a Bio-Gel column eluted with water was originally performed in order to desalt the AMannan and Mannan preparations prior to complete NMR analysis. In the absence of acetic acid in the elution buffer, a very clear separation of the Mannan components on Bio-Gel P6 in two distinctive mannose-containing fractions labelled Mannan1 and Mannan2 was achieved (Fig. 1b). Quantification by gas chromatography indicated that both fractions occurred in similar quantities (data not shown).

Structural determination of P-AMannan and P-Mannan

Monosaccharide and inositol composition of Mannan1 and Mannan2 were investigated by GC and clearly indicated that both fractions contained almost exclusively mannose

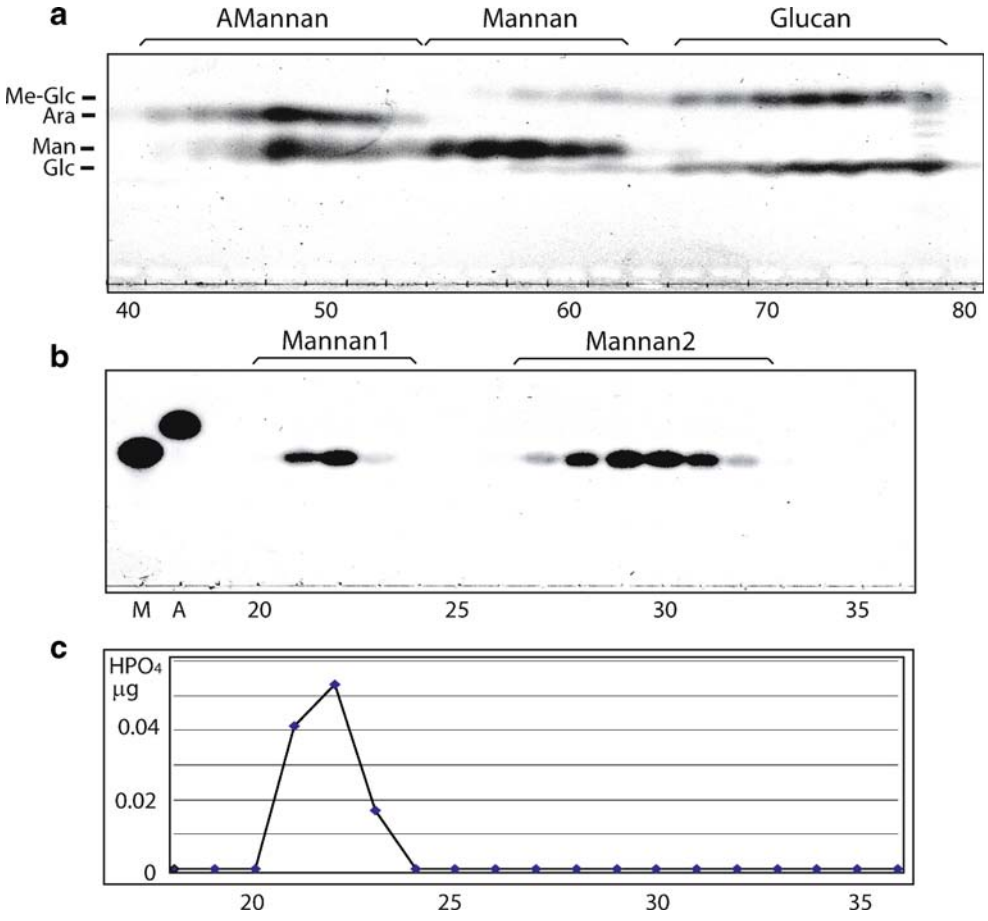

Fig. 1 Separation of polysaccharides. **a** Separation of the three classes of polysaccharides was achieved by chromatography using a Bio-Gel P10 column. Elution was performed using 0.1% acetic acid and fractions were analysed by TLC after complete hydrolysis with TFA. Standards of Me-Glc, Ara, Man and Glc were used and indicated in the left margin. Compositions of individual components were checked with GC/MS. **b** Mannans were further separated on a Bio-Gel P6 column following elution with water. Fractions were analysed by TLC after hydrolysis with TFA. Spots labelled "M" and "A" correspond to Man and Ara standards. **c** Presence of phosphate in the eluted fractions of the Bio-Gel P6 column was assayed by HPAEC

residues (Table 1). However, they differed by only the presence of a *myo*-inositol in Mannan1 in the Ins/Man ratio of 1:52. In order to determine whether the inositol residue was present at the reducing position of Mannan1, as found in the related LM, both Mannan1 and Mannan2 were reduced in presence of sodium borohydrate and methanolyzed. The resulting components were analysed by GC as trimethylsilyl derivatives. As shown in Table 1, reduction of Mannan2 generates a mannitol (Man-ol) residue in a Man/Man-ol ratio of 53:1, strongly suggesting the occurrence of a mannose residue in the reducing end of Mannan2. This observation is consistent with previous studies demonstrating the presence of Man residues in reducing positions of the related AMannans isolated from either *M. tuberculosis* or *M. bovis* BCG [2, 21]. On the contrary, reduction of Mannan1 generated only small amounts of Man-ol, suggesting that it does not possess a Man residue at the reducing end (Table 1).

We next examined the presence of a phosphate group associated to the inositol residue of Mannan, as observed in the related LM. The presence of phosphate was assessed by

Table 1 Monosaccharide composition of purified reduced polysaccharides (expressed in number of residues/molecule) was determined by GC

	Man	Ara	Ins	Man-ol
Mannan1	52,0	2,0	1,0	0,2
Mannan2	53,0	1,0	–	1,0
AraMannan1	40,0	35,0	1,0	0,1
AraMannan2	45,0	38,0	–	1,0

Compositions are standardized according to either Ins or Man-ol compositions

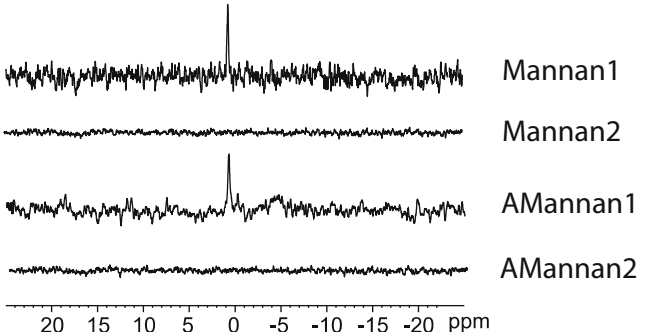

Fig. 2 Analysis of the phosphate content in the various *M. kansasii* glycoconjugates. The presence of phosphate was assessed in each polysaccharide by ^{31}P NMR spectrometry. Signal at δ 0.79 attributed to phosphate group of phosphatidyl-*myo*-inositol anchor was exclusively observed in Mannan1 and AMannan1

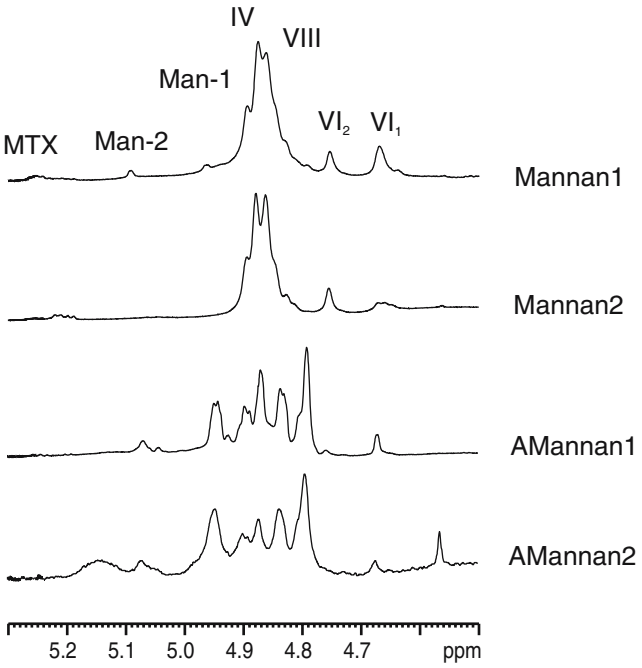

Fig. 3 Comparison of ^{1}H NMR parameters in the various *M. kansasii* glycoconjugates. Details of anomeric region of ^{1}H NMR spectra demonstrate that glycan moieties the two fractions of polysaccharides (Mannan1/2 and AMannan1/2) exhibited very similar features

HPAEC along with the elution profile of Mannans from gel filtration. It shows that phosphate was specifically associated to Mannan1-containing fractions but virtually absent from Mannan2 (Fig. 1c). This was further confirmed by ^{31}P-NMR that exclusively showed an intense ^{31}P signal at 0.79 ppm in Mannan1 but not in Mannan2 (Fig. 2). A similar set of data was obtained for the AMannan components. Separation by Bio-Gel P-10 chromatography AMannan generated two distinct fractions, namely AMannan1 containing an inositol residue (Ara/Man/Ins ratio of 40/35/1, Table 1) and a phosphate group (Fig. 2) whereas AMannan2 terminated with a mannose residue at its reducing end (Table 1, Fig. 2).

In order to determine the nature of the structural differences between fractions 1 and 2, all components were analysed by ^{1}H, ^{13}C and ^{31}P NMR with a special emphasis on the reducing positions of polysaccharides. All NMR parameters were determined by comparison with previous work analysis of the glycan moiety of LM and LAM from *M. kansasii* [13]. NMR parameters of the mannan and arabinan domains from both polysaccharides perfectly fit with those previously observed for LAM and LM, indicating that the neutral polysaccharides share identical glycan moieties with their lipoglycan equivalents. Briefly, mannan domains of both AMannans and Mannans are characterised by a chain of α1-6-linked mannose backbone substituted in α1-2 position by mannose residues, as demonstrated by the observation of signals attributed to 6-α-Man*p* (VI1 and VI2), 2-6-α-Man*p* (VIII) and t-α-Man*p* (IV) residues (Figs. 3 and 4). The presence of oligomannosylated side chains was not tested in the scope of the present study by chemical and immunological means as for their lipoglycans counterparts [13]. However, we confirmed the presence of 5-deoxy-5-methylthio-xylofuranose (MTX) residues in all polysaccharides owing to the observation of its anomeric signal at δ ^{13}C/^{1}H 103.9/5.23, as in lipoglycans of *M. kansasii*. This was confirmed by the observation of its entire spin system from ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC TOCSY experiments. This unusual substitution was found to be present in both *M. tuberculosis*

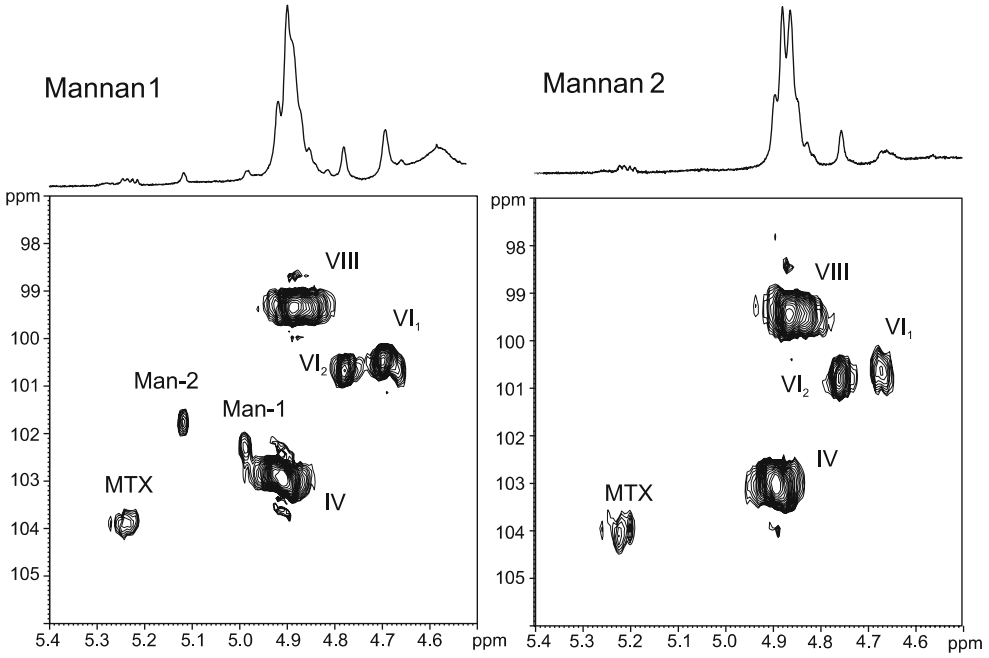

Fig. 4 Detail of HSQC ^{1}H/^{13}C NMR spectra of Mammans. Anomer region of glycan moieties of Mannan1 and Mannan2 only differ by the presence of mannose residues (Man-1 and Man-2) on inositol group

and *M. kansasii* lipoglycans, either attached to the mannose capping motifs for the former species or to the mannan domain for the latter [13, 22, 23]. The MTX absolute stereochemistry, linkage position and conformation have recently been described allowing to demonstrate that they present a D-configuration and are attached to a Man*p* residue *via* an α1-4-linkage [24]. Observation of MTX in both Mannans and AMannans confirmed the seminal observation that this residue substitutes the mannan domain in *M. kansasii*. Analysis of the NMR parameters of the arabinan domain of AMannan and LAM from *M. kansasii* were very similar, demonstrating that both glycoconjugate compounds also share identical arabinan domains (data not shown).

Structural determination of the of P-AMannan and P-Mannan reducing ends by NMR

Comparison of NMR data of fractions 1 and 2 from Mannan and AMannan confirmed the hypothesis that they only differ by the nature of their reducing end. As shown in Figs. 4 and 5, the anomeric regions of one homonuclear ^{1}H and heteronuclear $^{1}H/^{13}C$ NMR spectra of Mannan1 and Mannan2 are very similar, only differing by the presence in Mannan1 of two signals at δ 4.98/102.3 and 5.12/101.7 previously attributed to Man residues substituting non acylated inositol in C6 position (Man1) and C2 position (Man2), respectively [13, 25]. These two signals are usually associated with the phosphatidyl-*myo*-inositol anchor ubiquitously observed in LM. Their observation in Mannan1 demonstrates the presence of an inositol residue at the reducing end of the mannan domain, as suggested by above preliminary results. Accordingly, spin system of the inositol residue observed in a COSY 90 experiment (Fig. 5a and b) is identical to the non acylated form of inositol from phosphatidyl-*myo*-inositol observed in LM and LAM from *M. kansasii* [13]. Then ^{31}P-^{1}H HSQC and HSQC TOCSY experiments (Fig. 5c) demonstrated the presence of a phosphatidyl-*myo*-inositol motif through connections of phosphate group to Ins H1 and H2 and to three signals at δ 3.77, 3.53 and 3.36 tentatively attributed to H3,3′, H2 and H1,1′ of non acylated glycerol (Gro). Indeed, strong shielding compared to LM of Gro-H2 and Gro-H1,1′ from δ 5.1 and 4.35/4.12 to δ 3.53 and 3.36 is in agreement with deacylation at positions C2 and C1, respectively. This attribution was unambiguously confirmed by comparing Gro NMR parameters of native LM from *M. kansasii* and its deacylated equivalent. As shown in Fig. 5, removal of fatty acids from glycerol residue by saponification with NaOH induces a strong shielding of Gro-H1,1′ to δ 3.36, as observed in Mannan1. Further comparison of Ins and Gro spin systems from Mannan1 and deacylated LM demonstrated that both compounds shared non acylated phosphatidyl-*myo*-

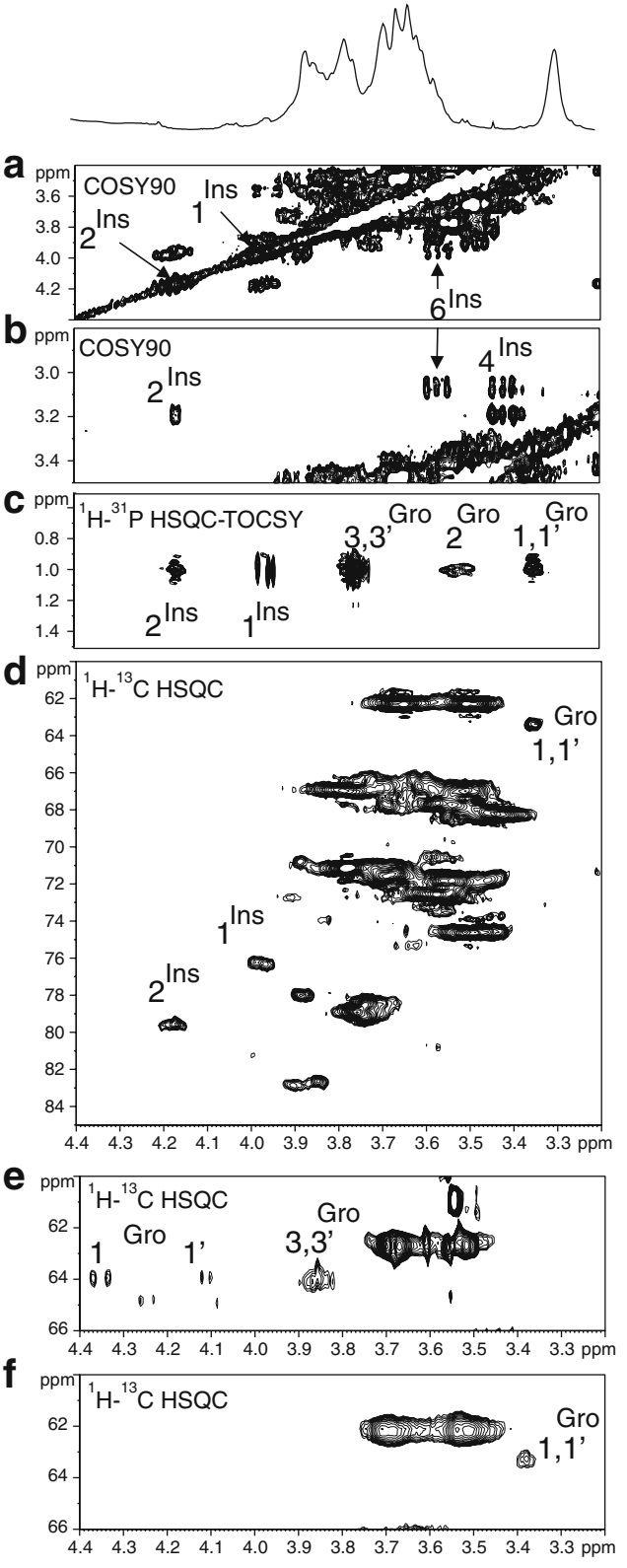

Fig. 5 Spin systems of inositol and glycerol from Mannan1. Details of **a**, **b** $^{1}H/^{1}H$ COSY 90 spectrum of Mannan1; **c**, ^{1}H-^{31}P HSQC-TOCSY spectrum of Mannan1 showing the complete spin system of Gro and partial spin system of Ins from phosphate; **d** ^{1}H-^{13}C HSQC spectrum of Mannan1; **e** ^{1}H-^{13}C HSQC spectrum of LM from *M. kansasii*; **f** ^{1}H-^{13}C HSQC spectrum of LM from *M. kansasii* after alkalynolysis

inositol anchors. In contrast, and in agreement with the composition analysis, no Gro and Ins spin systems were observed in Mannan2.

MALDI-MS analysis established that LM, Mannan1 and Mannan2 exhibited quite similar molecular weights (Fig. 6). As previously observed for LM, they all appear as polydisperse molecules with a calculated degree of polymerization ranging from about 25 to 54 for LM, 29 to 55 (maximum intensity for Hex_{42}) for Mannan1 and from 18 to 46 (maximum intensity for Hex_{38}) for Mannan2. This latter appears as slightly smaller than the two other components, which suggest that it may originate from processing of either LM or Mannan1 by endo-mannosidase degradation. In conclusion, compelling data from composition, MS and NMR analyses clearly establish that Mannan1 and Mannan2 shared identical glycan moieties but differ in the nature of the motif at the reducting end. Mannan1 is characterised by the presence of a non acylated phosphatidyl-*myo*-inositol anchor, whereas Mannan2 possesses a reducing mannose residue. Identical results were obtained from the comparison of NMR parameters from AMannan fractions. AMannan1 only differs from AMannan2 by the presence of a non-acylated phosphatidyl-*myo*-inositol anchor.

Surprisingly, (A)Mannan1 and (A)Mannan2 were easily separated by gel filtration whereas they exhibit very similar molecular weights. This very clear separation is likely to be the result of the ionic exclusion from the gel filtration

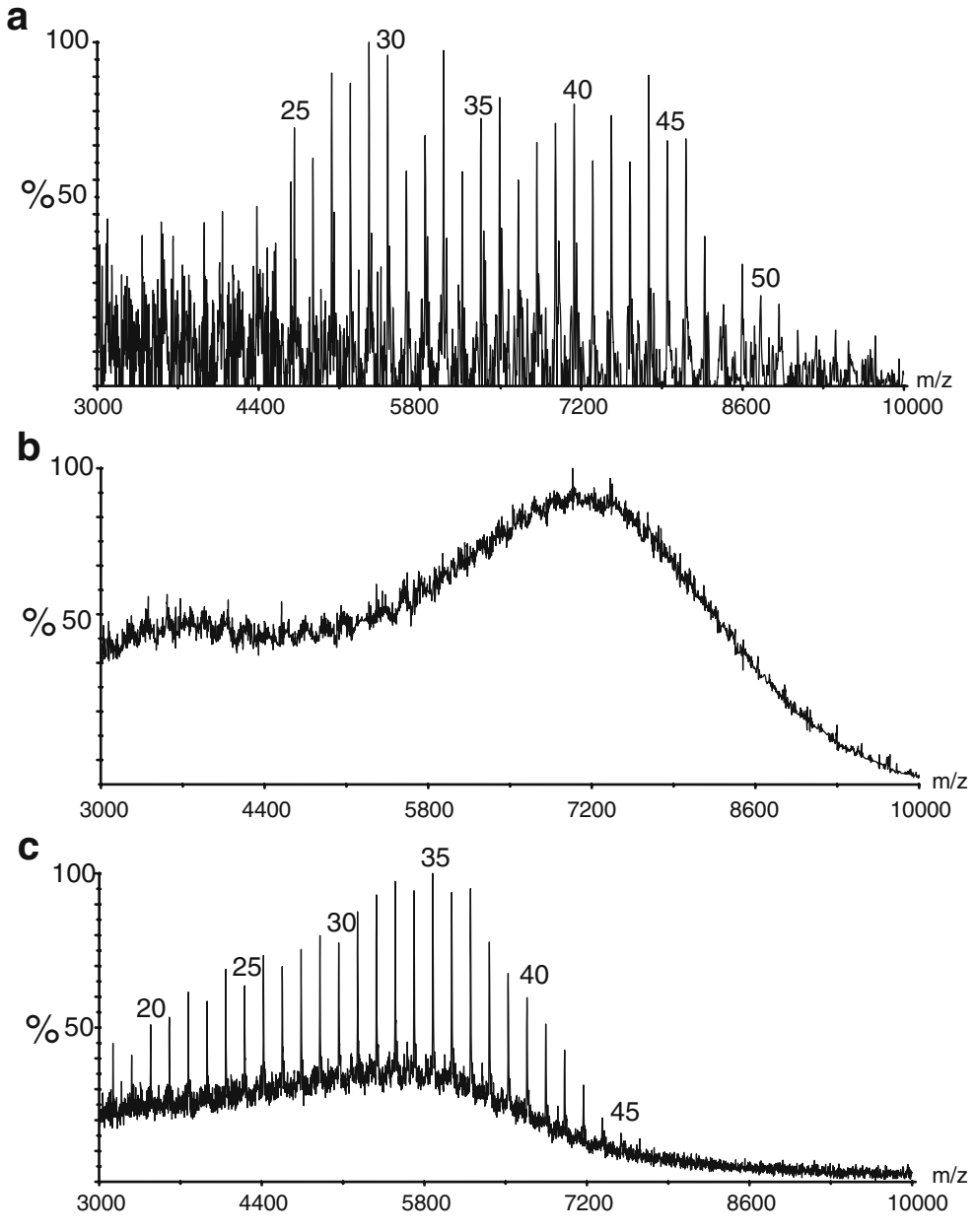

Fig. 6 MALDI-MS analysis of *M. kansasii* polysaccharides. **a** LM, **b** Mannan1, and **c** Mannan2. All compounds appear as polydisperse molecules with average molecular masses of 6,400, 6,240 and 5,300 mass units (mu), respectively. Degree of polymerization of the mannan chain for LM and Mannan2 was expressed in number of mannose residues. They were assessed according to the presence of di-acylated phosphatidyl-*myo*-inositol anchor or reducing mannose, respectively. MALDI-TOF analysis did not clearly differentiate glycol-forms for Mannan1 which MS spectrum appears as very broad peak with a maximum signal at m/z 7050±20

column of the negatively charged phosphorylated molecules. This phenomenon is often observed on polyacrylamide based gels when used with very low ion strength solvents due to the presence of residual negatively charged groups on the gels. By this simple means, we could distinguish the presence of phosphorylated glycoconjugates, whereas previous studies on capsular mannans and arabinomannans exclusively described polysaccharides with a reducing end that correspond to the Mannan2 and AMannan2 [2, 21]. Although it may be argued that (A) Mannan1 result from deacylation of LAM or LM during the purification procedure, this hypothesis seems very unlikely considering that both hydrophilic and lipoglycans are separated during phase partition in detergent at a very early stage of the extraction process.

Here, we demonstrate that, in *M. kansasii*, Mannan and AMannan are present in three different forms depending on the motif at the reducing end (Fig. 7): (a) substituted with an acylated phosphatidyl-*myo*-inositol anchor (LM and LAM), (b) substituted with a non-acylated phosphatidyl-*myo*-inositol anchor (P-Mannan and P-AMannan), and (c) with a free reducing end (Mannan and AMannan). The presence of high amounts of these new phosphorylated glycoconjugates, and considering that glycan moieties of LM/P-Mannan/Mannan and LAM/P-AMannan/AMannan are strictly identical, we hypothesize for a direct biosynthetic pathway linking each of these components. It is tempting to hypothesize that P-Mannan/P-AMannan derive directly from LM/LAM by removal of the fatty acids bound to the inositol or the glycerol of the GPI moiety, presumably by enzymatic cleavage. AMannan/Mannan would then directly originate from P-Mannan/P-AMannan by release of the phosphatidyl-*myo*-inositol group, through the action of another enzyme.

Although LM/LAM are primarily found to be associated to the cell wall, it was proposed that AMannan/Mannan are important components of the capsule surrounding mycobacteria [1]. P-AMannan and P-Mannan were isolated in the detergent-depleted phase (hydrophilic phase), during the Triton X-114 phase separation method, establishing their hydrophilic nature. This is in agreement with the GC and NMR data revealing the absence of fatty acids. Together, this strongly suggests that, like AMannan and Mannan, P-AMannan and P-Mannan are part of the capsule rather than the cell wall. However, the exact localization of P-AMannan and P-Mannan compared with their non-phosphorylated equivalents remains to be further investigated through the use of mild extraction methods of the capsular components [2]. It has been reported that AMannan is an immunologically important antigen [26] and that a monoclonal antibody, 9d8, that specifically binds to AMannan but not to LAM, prolonged the survival of mice infected with *M. tuberculosis* [4]. Whether this antibody recognizes also P-AMannan merits to be investigated. Analysis of the human antibody response to AMannan demonstrated that IgG and IgM to AMannan were detected in the serum samples of both patients and controls, with higher antibody titres in patients sera [27]. Given the capsular localization of Mannan, and its structural similarity with AMannan, it is anticipated that P-AMannan is an important polysaccharide antigen of the capsule and capable to generate a specific antibody response in tubercu-

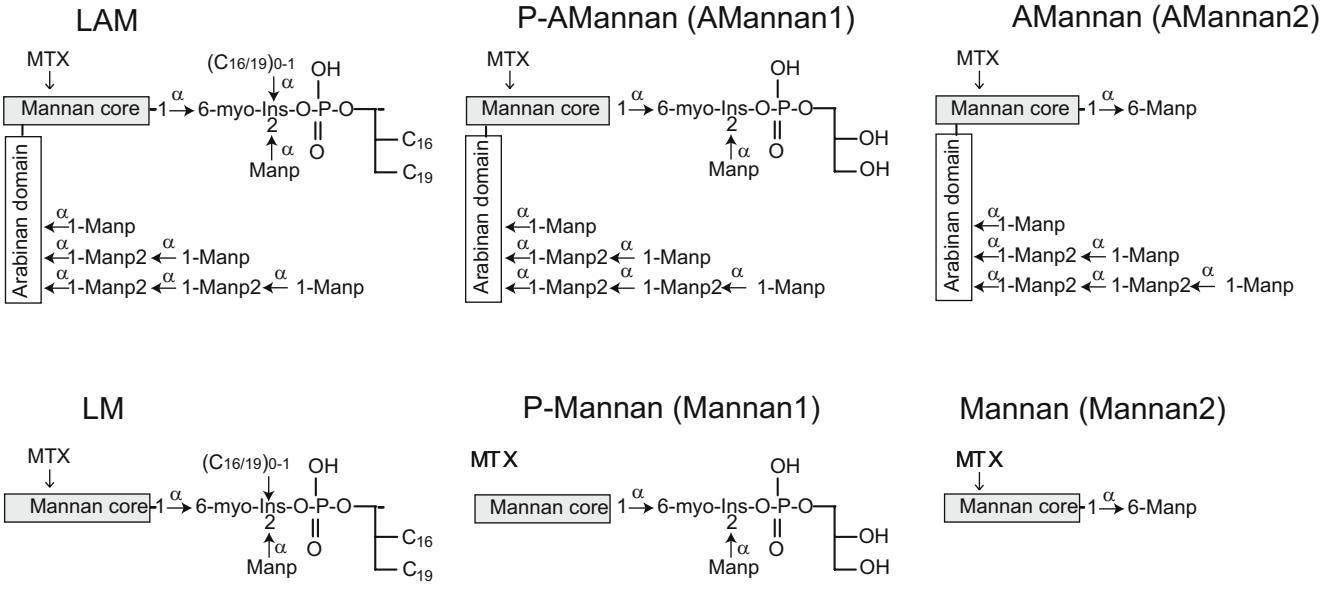

Fig. 7 Schematic representation of the various glycoconjugates from *M. kansasii*. The mannan core consists of a linear polymer of α1-6-linked mannose residues punctuated by α1-2-linked Man*p* residues. The arabinan domain consists predominantly of a linear polymer of α1-5-linked Ara*f* residues substituted by branching arabinosides. Terminal Ara*f* residues can bear mono-, di- or tri-mannooligosaccharides producing the capping motifs. MTX, 5-deoxy-5-methylthio-xylofuranose; Ins, inositol; Man*p*, mannopyranose; LAM, lipoarabinomannan; P-AMannan, phosphorylated arabinomannan; AMannan, arabinomannan; LM, lipomannan; P-Mannan, phosphorylated mannan

losis patients. However, whether AMannan and P-AMannan are antigenically different remains to be examined. Moreover, *M. tuberculosis* surface carbohydrates have been proposed to be important factors influencing the adherence of bacteria to a complement receptor of macrophages [28]. It is therefore possible that P-(A)Mannan may represent important surface polysaccharides interacting with host macrophages.

This study presents new insights into the structural variability of mycobacterial polysaccharides and P-AMannan/P-Mannan represent powerful tools to investigate the role of glycosyl-phosphatidyl inositol acylation in various biological functions assigned to LAM/LM. Indeed, the need to the acyl residues to maintain the functional integrity of the ManLAM has been previously put forward to explain the lack of interaction of deacylated LAM with host immune cells [29, 30] as well as its inability to modulate the phagocyte cytokine production [31, 32]. Moreover, the fatty acid requirement also seems to be critical for a direct binding between LAM and human pulmonary surfactant protein A [33]. Therefore, P-AMannan/P-Mannan could be added along with LAM/LM to directly address the role of the fatty acid in various assays such as cytokine production, formation of multigiant cells, or interaction with cell surface receptors. In this regard, we have recently used these molecules to analyse the binding kinetics of LM/LAM from *M. kansasii* to CD14 and LPS-binding protein (LBP) by surface plasmon resonance and found that neither P-AMannan nor P-Mannan were capable of interacting with these two surface receptors, indicating that the fatty acids of LM/LAM are indispensable for a direct interaction with CD14 and LBP [34]. Recently, the importance of the acylation state of LM with respect to TLR2-mediating activation of macrophages was described [11]. Therefore, expression of non-acylated phosphorylated P-AMannan/P-Mannan along with their corresponding acylated LM/LAM counterparts in the whole bacteria, may have an important impact with regard to the physiopathology and control of mycobacterial infections. Although it remains to be investigated, we hypothesize that the P-AMannan/LAM and the P-Mannan/LM ratios are important factors that may influence the outcome of the infection.

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