Lipoarabinomannans: characterization of the multiacylated forms of the phosphatidyl-*myo*-inositol anchor by NMR spectroscopy

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Lipoarabinomannans, which exhibit a large spectrum of immunological activities, emerge as the major antigens of mycobacterial envelopes. The lipoarabinomannan structure is based on a phosphatidyl-*myo*-inositol anchor whose integrity has been shown to be crucial for lipoarabinomannan biological activity and particularly for presentation to CD4/CD8 double-negative $\alpha\beta$ T cells by CD1 molecules. In this report, an analytical approach was developed for high-resolution ³¹P-NMR analysis of native, i.e. multiacylated, lipoarabinomannans. The one-dimensional ³¹P spectrum of cellular lipoarabinomannans, from *Mycobacterium bovis* Bacillus Calmette–Guérin, exhibited four ³¹P resonances typifying four types of lipoarabinomannans. Two-dimensional ¹H-³¹P heteronuclear multiple-quantum-correlation/homonuclear Hartmann–Hahn analysis of the native molecules showed that these four types of lipoarabinomannan

differed in the number and localization of fatty acids (from 1 to 4) esterifying the anchor. Besides the three acylation sites previously described, i.e. positions 1 and 2 of glycerol and 6 of the mannosyl unit linked to the C-2 of *myo*-inositol, we demonstrate the existence of a fourth acylation position at the C-3 of *myo*-inositol. We report here the first structural study of native multiacylated lipoarabinomannans, establishing the structure of the intact phosphatidyl-*myo*-inositol anchor. Our findings would help gain more understanding of the molecular basis of lipoarabinomannan discrimination in the binding process to CD1 molecules.

Key words: acylation sites, Bacillus Calmette–Guérin, heteronuclear correlation, lipoglycan, phosphorous.

INTRODUCTION

Tuberculosis still kills more people each year than any other single infectious disease [1]. Lipoarabinomannans (LAMs), found ubiquitously in the genus *Mycobacterium* [2], represent the major antigens modulating immune responses of the host [3,4]. For example, they have been shown to regulate cytokine secretion [5–12], block the transcriptional activation of interferon- γ [13] and neutralize the potentially cytotoxic oxygen free radicals [13]. In addition, mannosylated LAMs (ManLAMs) selectively bind murine and human macrophages via the mannose receptor [14,15]. It is now clearly demonstrated that most of these functions are abrogated with alkali-treated molecules, pointing to the importance of the alkali-labile residues. To date, the following alkali-labile residues have been identified: fatty acids [11,16,17], succinic acids [16,18] and phosphoinositides [9].

In addition, ManLAMs from *Mycobacterium leprae* and *M. tuberculosis* Erdman strains were found to be presented by CD1 molecules and stimulate CD4/CD8 double-negative $\alpha\beta$ T cells [19]. The recently established crystal structure of mouse CD1d1, corresponding to human CD1d, exhibited a hydrophobic binding site [20]. Moreover, cellular glycosylphosphatidylinositol was identified as the major natural ligand of mouse CD1d1 [21]. These results are consistent with observations that human CD1b and CD1c can present the amphipathic lipoarabinomannans but also point to the central role played by the ManLAM phosphatidylinositol moiety in the binding process to CD1.

LAM structure is based on a phosphatidyl-*myo*-inositol unit and may be a multiglycosylated form of lipomannans (LMs) and

the well-described phosphatidyl-*myo*-inositol mannosides (PIMs) [22–24]. The multiacylated nature of PIMs and LMs has been recently re-examined by others [17,22] and us (M. Gilleron, J. Nigou, B. Cahuzac and G. Puzo, unpublished work) in an attempt to find out, by structure homologies, whether they are biosynthetic precursors of LAMs. Khoo et al. [22] have unambiguously established the multiacylated nature of PIMs, LMs and LAMs using destructive techniques; nevertheless, in their report the number and the localization of the acyl residues or the proportion of the different phosphatidyl-*myo*-inositol anchor (PI anchor) forms was not completely demonstrated.

In a previous study, we showed that PI anchors played a key role in the stimulation of cytokine secretion from human dendritic cells by ManLAMs from *M. bovis* Bacillus Calmette–Guérin (BCG) [11]. Indeed, we found that the structurally different parietal and cellular ManLAM PI anchors differentially stimulate interleukin-8 and tumour necrosis factor- α secretion. In this study, the cellular PI anchor was only partially established by chemical-degradation studies based on alkaline hydrolysis and acetolysis. We demonstrated a marked heterogeneity in the phosphatidyl unit of cellular ManLAMs due to a combination of mono- and diacylglycerols. Native multiacylated cellular ManLAMs appeared to be inaccessible to NMR analysis, although one-dimensional (1D) ³¹P- and two-dimensional (2D) ¹H-³¹P-NMR provides a fast and non-destructive way of determining the structure of the PI anchor from lipoglycans including LAMs, i.e. glycerol acylation and inositol substitution. However, this approach is only effective for the deacylated LAMs [9,25,26] or lyso-lipoglycans [11], and is not useful for LAMs, with

Abbreviations used: Ac_nManLAM, ManLAM with *n* fatty acids; BCG, Bacillus Calmette–Guérin; DMF, dimethylformamide; DOC, sodium deoxycholate; HMQC, heteronuclear multiple-quantum-correlation; HOHAHA, homonuclear Hartmann–Hahn; LAM, lipoarabinomannan; LM, lipomannan; ManLAM, mannosylated LAM; $\nu_{1/2}$, linewidth; PI anchor, phosphatidyl-*myo*-inositol anchor; PIM, phosphatidyl-*myo*-inositol mannoside; TEA, triethylamine; 1D, one-dimensional; 2D, two-dimensional; Man_p, mannopyranose.

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multiacylated anchors, solubilized in $^2\text{H}_2\text{O}$ [11]. Indeed, in $^2\text{H}_2\text{O}$, the phosphorus from lipoglycans resonates as a broad unresolved signal due to aggregation of the amphipathic lipoglycans [27].

We describe here a solvent system for high-resolution ^{31}P -NMR analysis of the native multiacylated ManLAM molecules. Moreover, using 2D ^1H - ^{31}P - and ^1H - ^1H -NMR experiments, we established the number and the localization of the fatty acids on the cellular ManLAM PI anchor from *M. bovis* BCG. These structural findings will be discussed in terms of binding to CD1 and recognition by human double-negative $\alpha\beta\text{T}$ cells.

MATERIALS AND METHODS

Extraction and purification of cellular ManLAMs

Cellular ManLAMs were purified as described previously [11]. Briefly, *M. bovis* BCG Pasteur-strain cells were delipidated with $\text{CHCl}_3/\text{CH}_3\text{OH}$, 1:1 (v/v). The delipidated mycobacteria were extracted six times by refluxing in 50% ethanol at 65 °C for 8 h (parietal fraction). The resulting cells (cellular fraction) were washed and disintegrated in ice by sonication (20 kHz, 600 W, 15 min) and using a French pressure cell (one step of lysis at a pressure maintained above 200 MPa). Most of the proteins of the cellular fraction were removed by a hot 80% (w/w) aqueous phenol biphasic wash at 70 °C for 1 h. After enzymic treatments with trypsin, α -amylase, RNase A (from bovine pancreas; Sigma, Saint-Quentin Fallavier, France) and DNase I (from bovine pancreas; Fluka, Saint-Quentin Fallavier, France), the amino acids, glucose, nucleotides and phenol were removed from the cellular glycans and lipoglycans by dialysis. The glycan and lipoglycan mixtures were subjected to Triton X-114 phase separation and the ManLAMs and LMs were then separated by gel filtration. Samples (60 mg) were dissolved in 0.2 M NaCl/0.25% (w/v) sodium deoxycholate (DOC)/1 mM EDTA/10 mM Tris (pH 8) at a final concentration of 200 mg/ml, incubated for 2 days at room temperature and loaded on a gel-permeation Bio-Gel P-100 column (52 $\times$ 3 cm) eluted with the same buffer at a flow rate of 5 ml/h.

NMR spectroscopy

Deuteriated solvents, $^2\text{H}_2\text{O}$ (^2H , 99.97%), DMSO- $^2\text{H}_6$ (^2H , 99.8%) and dimethylformamide (DMF)- $^2\text{H}_7$ (^2H , 99.5%), were from Euriso-top (Saint-Aubin, France). L- α -Lysophosphatidyl-inositol, i.e. monoacyl-*sn*-glycero-3-phospho-(1-*D*-*myo*-inositol) standard, was purchased from Sigma and was a mixture of approximately 82% 1-acyl-2-lyso-*sn*-glycero-3-phospho-(1-*D*-*myo*-inositol) (determined by integration of the ^{31}P signal at 5.39 ppm obtained in DMSO- $^2\text{H}_6$ at 343 K) and 18% 1-lyso-2-acyl-*sn*-glycero-3-phospho-(1-*D*-*myo*-inositol) (^{31}P signal at 4.94 ppm).

NMR spectra were recorded on a Bruker AMX-500 spectrometer equipped with an Aspect X32 computer. Samples were exchanged in $^2\text{H}_2\text{O}$ with intermediate freeze-drying, then dissolved in $^2\text{H}_2\text{O}$ or in detergents (Triton X-114, Triton X-100, DOC) with $^2\text{H}_2\text{O}$ or in DMF- $^2\text{H}_7$ / $^2\text{H}_2\text{O}$ (4:0.6, v/v), DMSO- $^2\text{H}_6$ or DMSO- $^2\text{H}_6$ /triethylamine (TEA; 9:1, v/v) and analysed in 200 $\times$ 5 mm 535-PP NMR tubes. The concentration of the NMR samples were 120 mg/ml in $^2\text{H}_2\text{O}$, 20 mg/ml in 1% DOC/100 mM EDTA and 54 mg/ml in DMF- $^2\text{H}_7$ / $^2\text{H}_2\text{O}$ (4:0.6, v/v), DMSO- $^2\text{H}_6$ and DMSO- $^2\text{H}_6$ /TEA (9:1, v/v). Spectra were recorded at 313, 323 and 343 K.

The 1D proton (^1H) spectra were measured using a 90° tipping angle for the pulse and 1 s as the recycle delay between each of the eight acquisitions of 1.64 s. The spectral width of 4505 Hz in $^2\text{H}_2\text{O}$ and 3378 Hz in DMSO- $^2\text{H}_6$ was collected in 16000 complex

points that were multiplied by a Gaussian function (line-broadening factor, -1; Gaussian-broadening factor, 0.2) prior to processing to 32000 real points in the frequency domain. After Fourier transformation, the spectra were base-line corrected with a fourth-order polynomial function. The ^1H -NMR chemical shifts were referenced to the internal acetone signal at 2.225 ppm for experiments conducted in $^2\text{H}_2\text{O}$ and to the internal DMSO signal at 2.52 ppm for the experiments conducted in DMSO- $^2\text{H}_6$.

The 1D phosphorus (^{31}P) spectra were measured at 202.46 MHz using phosphoric acid (85%) as external standard (δ_{P} , 0.0). The data were collected in 16000 complex data sets, and an exponential transformation (line-broadening factor is detailed in the Figure legends) was applied prior the processing to 64000 real points in the frequency domain. The ManLAM concentrations, scan numbers and spectral widths are indicated in the Figure legends.

All 2D spectra were recorded without sample spinning and data were acquired in the phase-sensitive mode using the time-proportional phase increment method [28]. The 2D ^1H - ^1H homonuclear Hartmann-Hahn (HOHAHA) spectra in DMSO- $^2\text{H}_6$ were recorded using a MLEV-17 mixing sequence of 115 ms [29]. The spin-lock field strength corresponded to a 90° pulse with 30 μs . The spectral width was 3153 Hz in the F_2 dimension and 2025 Hz in the F_1 dimension: 248 spectra of 4096 data points with 80 scans/ t_1 increment were recorded (where t_1 is evolution time). Data were processed as above.

The 2D ^1H - ^{31}P -heteronuclear multiple-quantum-correlation (HMQC) and ^1H - ^{31}P -HMQC-HOHAHA spectra were recorded in the proton-detection mode with a Bruker 5 mm ^1H broad-band tunable probe with reversal geometry. The globally optimized alternating-phase rectangular pulse (GARP) sequence [30] at the phosphorus frequency was used as a composite pulse decoupling during acquisition. The single-bond HMQC spectrum was obtained according to Bax and Subramanian's pulse sequence [31]. Spectral widths of 2025 Hz in ^{31}P and 3153 Hz in ^1H dimensions were used to collect a 4096 $\times$ 80-point data matrix (time-proportional phase increment) with 120 scans/ t_1 value expanded to 4096 $\times$ 1024 by zero filling. The relaxation delay was 1 s. A sine-bell window shifted by $\pi/2$ was applied in both dimensions. An HMQC-HOHAHA spectrum was obtained using the Lerner and Bax pulse sequence [32]. Spectral widths of 2025 Hz in ^{31}P and 3376 Hz in ^1H dimensions were used to collect a 4096 $\times$ 78-point data matrix (time-proportional phase increment) with 376 scans/ t_1 value expanded to 4096 $\times$ 1024 by zero filling. The relaxation delay was 1 s and the mixing time was 53 ms. A sine-bell window shifted by $\pi/2$ was applied in both dimensions.

RESULTS

High-resolution ^{31}P -NMR of multiacylated ManLAMs

The cellular ManLAM 1D ^{31}P spectrum in $^2\text{H}_2\text{O}$ at 313 K exhibited a broad unresolved signal with a width of approximately 150 Hz (Figure 1a). This phenomenon is due to aggregate formation resulting from asymmetry in polarity of these molecules, which is also characteristic of phospholipids. Two techniques are currently employed for ^{31}P -NMR analysis of phospholipids, solubilization in water with detergents or solubilization in organic-solvent systems [33]. For the ^{31}P -NMR analysis of ManLAMs, we tested both methods of solubilization.

Figure 1(b) shows a 1D ^{31}P -NMR spectrum, recorded at 323 K, of ManLAMs dissolved in 1% (w/v) DOC/100 mM EDTA at pH 7. The spectrum exhibited two sharp resonances, Pa and Pb, at 0.27 and 0.53 ppm respectively, with linewidths ($\nu_{1/2}$) of 12 and 16 Hz respectively. The resolution, calculated as

Figure 1 1D ^{31}P spectra ($\delta^{31}\text{P}$, -3.50 to 4.50) of cellular ManLAMs dissolved in $^2\text{H}_2\text{O}$ (a), 1% DOC/100 mM EDTA, pH 7 (b) or DMSO- $^2\text{H}_6$ (c)

The spectra were recorded at (a) 313 K (120 mg/ml ManLAM, scan number, ns, 512), (b) 323 K (20 mg/ml, 1000 ns) and (c) 343 K after sonication for 20 min of the sample (54 mg/ml ManLAM, 128 ns). Spectral width was 25 kHz in all cases. An exponential multiplication (line-broadening factor = 5 Hz) was applied before Fourier transformation. (a) Phosphorus resonated at $\delta^{31}\text{P}$ 0.56 with ν_2 150 Hz; (b) Pa ($\delta^{31}\text{P}$ 0.27; ν_2 12 Hz), Pb ($\delta^{31}\text{P}$ 0.53; ν_2 16 Hz); (c) P1 ($\delta^{31}\text{P}$ 1.66; ν_2 13 Hz), P3 ($\delta^{31}\text{P}$ 1.83; ν_2 12 Hz).

$(|\nu_{\text{Pb}} - \nu_{\text{Pa}}|)/[(\nu_{1/2\text{Pb}} + \nu_{1/2\text{Pa}})/2]$, was 4.3. Despite sonication, heating and addition of detergent, we were unable to characterize the substituents esterifying Pa and Pb by 2D ^1H - ^{31}P experiments. Nevertheless, for the first time on native multiacylated ManLAMs, well-resolved ^{31}P resonances were observed, revealing the presence of two phosphate resonances, probably due to an heterogeneity in acylation state, since deacylated ManLAMs only exhibited a single ^{31}P resonance [11].

Bosco et al. [33] found TEA/DMF/guanidinium to be the most efficient solvent system for ^{31}P -NMR analysis of phospholipid mixtures, i.e. for chemical-shift dispersion of the ^{31}P signals, stability of the solvent and the possibility of realizing 2D experiments for structural elucidation. Likewise, ManLAMs were tentatively dissolved in DMF, but water was needed for a complete solubilization; DMF- $^2\text{H}_7$ / $^2\text{H}_2\text{O}$ (4:0.6; v/v). In this case, the 1D ^{31}P spectrum recorded at 343 K (results not shown) was dominated by one intense and sharp signal at 1.53 ppm with a linewidth of 22 Hz. For the first time on multiacylated LAMs,

Figure 2 1D ^{31}P spectra ($\delta^{31}\text{P}$, 1.00–4.00) of cellular ManLAMs (a) and cellular LMs (b) dissolved in DMSO- $^2\text{H}_6$

The spectra were recorded at 343 K after sonication for 20 min of the sample. (a) 54 mg/ml ManLAM, scan number, ns, 1662, 4052 Hz spectral width; (b) 40 mg/ml LM, 1024 ns, 25 kHz spectral width. An exponential multiplication (line-broadening factor = 2 Hz) was applied before Fourier transformation.

we were able to characterize the phosphate substituents from 2D ^1H - ^{31}P -HMQC and HMQC-HOHAHA experiments. However, using detergents, we previously obtained evidence for two populations of phosphate, Pa and Pb. Although the resolution of phosphate signals can be improved by addition of a basic compound such as TEA or pyridine [33,34], we wished to avoid deacylation and so did not attempt this on our solution of ManLAMs in DMF/water.

As indicated above, a small amount of water in DMF was required to solubilize ManLAMs, so we investigated the more polar solvent DMSO. The 1D ^{31}P spectrum (Figure 1c), recorded at 343 K after sonication of the sample, exhibited two signals, P1 and P3, at 1.66 and 1.83 ppm respectively. As the resolution of 2.7 was somewhat lower than that obtained using 1% DOC/100 mM EDTA as a solvent (4.3), we added TEA in the ratio DMSO- $^2\text{H}_6$ /TEA = 9:1 (v/v) in an attempt to enhance the ^{31}P chemical-shift dispersion. The P1 and P3 resonances in the 1D ^{31}P spectrum recorded at 343 K (results not shown) were shifted upfield to 1.39 and 1.56 ppm respectively, but the resolution was not affected. DMSO- $^2\text{H}_6$ was thus selected as the solvent for ^{31}P -NMR studies of ManLAMs, and the NMR parameters were optimized. Figure 2(a) shows the ^{31}P spectrum of cellular ManLAMs recorded at 343 K with an increased scan number. Besides P1 and P3, which remained the strongest resonances, three additional weak signals, P2, P4 and P5, were obtained at

Table 1 ^{31}P Chemical shifts and integration values of P1, P2, P3, P4 and P5 phosphates measured in $\text{DMSO-}^2\text{H}_6$ at 343 K

An integration value of 1.0 was fixed for the largest peak P1 + P2.

Phosphate ...	P1	P2	P3	P4	P5
δ (ppm)	1.66	1.72	1.83	3.33	3.50
Peak integration		1.0	0.85	0.12	0.15

1.72, 3.33 and 3.50 ppm respectively in the ratio P1/P2/P3/P4/P5 = 1:1:0.85:0.12:0.15 (Table 1). The ^{31}P resonances obtained could be classified in two groups according to their chemical shift: high field, P1, P2 and P3; and low field, P4 and P5.

In a previous study, we noted a marked heterogeneity in the phosphatidyl unit of cellular ManLAMs due to a combination of mono- and diacylglycerols [11]. Since the phosphate resonance of

a lyso-phospholipid (phospholipids with only one fatty acid on glycerol) has been shown to be downfield-shifted relative to the phosphate resonance of the same diacylated phospholipid [33,35], we tentatively assigned P1, P2 and P3 to phosphates from diacylphosphoglycerol units and P4 and P5 to phosphates from monoacylphosphoglycerol units. The 1D ^{31}P spectrum of cellular LMs from *M. bovis* BCG recorded in $\text{DMSO-}^2\text{H}_6$ at 343 K (Figure 2b) exhibited identical resonances to those observed for cellular ManLAMs and with similar intensities.

Structural determination of cellular ManLAM PI anchor

The proton resonances of the residues esterifying the different phosphates, i.e. *myo*-inositol and glycerol, could be assigned from the 2D experiments, $^1\text{H-}^{31}\text{P}$ -HMQC and -HMQC-HOHAHA, $^1\text{H-}^1\text{H}$ -HOHAHA, performed on ManLAMs dissolved in $\text{DMSO-}^2\text{H}_6$.

The $^1\text{H-}^{31}\text{P}$ -HMQC-HOHAHA spectrum of cellular ManLAMs dissolved in $\text{DMSO-}^2\text{H}_6$ (Figure 3a) exhibited a

Figure 3 2D $^1\text{H-}^{31}\text{P}$ -HMQC-HOHAHA mixing time 53 ms (a), $^1\text{H-}^{31}\text{P}$ -HMQC (b), $^1\text{H-}^1\text{H}$ -HOHAHA mixing time 115 ms (c, d) spectra of cellular ManLAMs dissolved in $\text{DMSO-}^2\text{H}_6$

Expanded regions (a, b) (δ ^1H , 2.90–5.30; δ ^{31}P , 0.50–4.50), (c) (δ ^1H , 2.90–5.30 and 2.98–3.27), and (d) (δ ^1H , 2.90–5.30 and 4.23–4.45) are shown. The spectra were recorded at 343 K after sonication for 20 min of the sample (54 mg/ml ManLAM). Numerals correspond to the proton number of the *myo*-inositol units; and numerals with letter G, to the proton number of the glycerol units. ●, indicates connectivities present on spectra but with weak intensity; connectivities with ■ (c) do not belong to the *myo*-inositol system although they are on the 5(P3) correlation line. Indeed, they are not on P3 (a) and 3(P3) at $\delta_{\text{H-3}}$ 3.24 in F_2 (c) lines. 5(P3) and 5(P1) correlation lines in HOHAHA spectrum (c) are partially superimposed with the 5(P5) and 5(P4) lines respectively, but these last two lines were too faint to be identified.

Table 2 Proton chemical shifts of *myo*-inositol and glycerol units from ManLAM PI anchors characterized by P1, P3, P4 and P5 phosphates

Chemical shifts are measured in DMSO- d_6 at 343 K, expressed in ppm, and referenced relative to internal DMSO signal at 2.52 ppm. nd, not determined.

	<i>myo</i> -Inositol						Glycerol		
	H-1	H-2	H-3	H-4	H-5	H-6	H-1/H-1'	H-2	H-3/H-3'
P1	4.09	4.20	4.59	3.65	3.16	3.65	4.32/4.10	5.10	3.84/3.80
P3	4.03	4.15	3.24	3.45	3.10	3.60	4.35/4.13	5.13	3.86/3.81
P4	4.09	4.24	4.59	3.65	3.17	3.65	3.97	nd	3.77
P5	4.00	4.18	3.24	3.44	3.10	3.61	4.00	nd	3.79

complex pattern of correlation peaks for each phosphate resonance. According to data in the literature [34], the downfield resonances at δ 5.13 and 5.10 in the F_2 dimension could be assigned to methine proton H-2 of a diacylated glycerol unit. These resonances were only observed for P1 and P3; no correlations corresponding to these protons were detected for the P4 and P5 resonances. These data supported the assumption that only P1, P3, and probably P2, belong to 1,2-diacyl-3-phospho-*sn*-glycerol units. P2 *myo*-inositol and glycerol spin systems were not characterized in cellular ManLAMs, because of the low intensity of P2 and partial superimposition on P1, although they were partially identified in the cellular LMs (M. Gilleron, J. Nigou, B. Cahuzac and G. Puzo, unpublished work). Starting from the proton resonance at δ 5.13, the glycerol spin system corresponding to P3 was defined from the HOHAHA spectrum (Figure 3d): δ 4.35, 4.13, 3.86 and 3.81 (Table 2). By means of the ^1H - ^{31}P -HMQC spectrum (Figure 3b) resonances at δ 3.86 and 3.81 were attributed to H-3 and H-3', and consequently, the remaining δ 4.35 and 4.13 to H-1 and H-1', which is consistent with a diacylated glycerol unit. The *myo*-inositol spin system belonging to the PI anchor characterized by P3 was identified from the correlation line at δ 3.10 (in the F_1 dimension) in the HOHAHA spectrum (Figure 3c). The resonance at δ 4.03 was attributed to H-1 from the ^1H - ^{31}P -HMQC experiment (Figure 3b). From their chemical shift, the multiplicity of the signals and the $^3J_{\text{HH}}$ coupling constants, the five remaining protons were assigned, according to data in the literature [25] and in comparison with a standard [*sn*-glycero-3-phospho-(1-*D*-*myo*-inositol), $\delta_{\text{H-1}}$ 3.65 (pd), $\delta_{\text{H-2}}$ 3.88 (ps), $\delta_{\text{H-3}}$ 3.18 (pd), $\delta_{\text{H-4}}$ 3.44 (t), $\delta_{\text{H-5}}$ 2.99 (t) and $\delta_{\text{H-6}}$ 3.66 (t); where pd is pseudodoublet, ps is pseudosinglet and t is triplet]. The δ 4.15, 3.60, 3.45, 3.24 and 3.10 signals were assigned to H-2, H-6, H-4, H-3 and H-5 respectively (Table 2). In a previous study [11], the anchor structure was shown to be characterized by glycosylation of the *myo*-inositol at C-2 by one mannopyranose (Manp) unit and at C-6 by the mannan core. Taken together, these results indicated that the PI anchor characterized by P3 shared a diacylated glycerol unit and a *myo*-inositol unit glycosylated at positions C-2 and C-6. The glycerol and *myo*-inositol spin systems belonging to a PI anchor characterized by P1 were analysed in a similar way. Starting from the independently resonating signal at $\delta_{\text{H-2}}$ 5.10 (Figure 3a), the glycerol H-1, H-1', H-3 and H-3' were assigned to, respectively, the δ 4.32, 4.10, 3.84 and 3.80 resonances (Table 2) in the HOHAHA (Figure 3c) and HMQC (Figure 3b) spectra. The *myo*-inositol spin system belonging to P1 was identified from the correlation line at $\delta_{\text{H-5}}$ 3.16 (in the F_1 dimension) in the HOHAHA spectrum (Figure 3c). δ 4.09 was assigned to H-1 from the HMQC spectrum (Figure 3b). The remaining *myo*-inositol H-3, H-2, H-6, H-4 and H-5 protons

Figure 4 1D ^1H spectra of cellular ManLAMs dissolved in $^2\text{H}_2\text{O}$ (a) and DMSO- d_6 (b, c)

Expanded regions (a) (δ ^1H , 4.45–5.55), (b) (δ ^1H , 4.25–5.35) and (c) (δ ^1H , 4.25–4.65) are shown. The spectra were recorded at 313 K (120 mg/ml ManLAM, scan number, ns, 8) (a) and 343 K (54 mg/ml, 8 ns) (b, c). A Gaussian multiplication (line-broadening factor, —1; Gaussian-broadening factor, 0.2) was applied before Fourier transformation. I, 3,5-*O*-Araf; II, 5-*O*-Araf; III, 2-*O*-Araf; IV, 1- α -Manp; V, 1- β -Araf; VI, 6-*O*-Araf; VII, 2-*O*-Manp; VIII, 2,6-*O*-Manp. These resonances were assigned from the ^1H - ^{13}C -HMQC, -HMQC-HOHAHA and ^1H - ^1H -HOHAHA experiments (results not shown). HOD, $^2\text{H}_2\text{O}$.

were assigned from the multiplicity of the signals and the $^3J_{\text{HH}}$ coupling constants at δ 4.59, 4.20, 3.65, 3.65 and 3.16 resonances respectively. The pseudodoublet at δ 4.59 was unambiguously assigned to H-3 according to the vicinal coupling constants $^3J_{\text{H-3,H-4}} = 10.7$ Hz and $^3J_{\text{H-2,H-3}} = 2.1$ Hz. The H-3 deshielding resonance ($\Delta\delta + 1.35$ ppm) compared with the one in the P3 *myo*-inositol spin system suggested that in this case, *myo*-inositol was acylated on C-3. This attribution is in agreement with data obtained on LMs and the tetra-acylated forms of PIM₂ from *M. bovis* BCG (M. Gilleron, J. Nigou, B. Cahuzac and G. Puzo, unpublished work). The H-4 resonance appeared to be affected by this acylation on C-3 since it was downfield-shifted by 0.20 ppm in comparison with that for P3. These observations demonstrated that the PI anchor characterized by P1 consisted of a diacylated glycerol and a *myo*-inositol glycosylated on C-2 and C-6 and acylated on C-3.

The P4 and P5 *myo*-inositol spin systems were assigned by comparison with those of P1 and P3. P4 exhibited a similar *myo*-inositol spin system to that of P1, while that of P5 was akin to the P3 system (Figure 3a, Table 2). The H-1 chemical shifts were deduced from the HMQC spectrum (Figure 3b). In both cases, only the *myo*-inositol H-2 resonances were slightly deshielded compared with the P1 and P3 *myo*-inositol H-2 resonances. The glycerol spin systems were attributed from the HMQC spectrum, revealing H-3 and H-3' resonances at δ 3.77 in P4 and at δ 3.79 in P5, and in comparison with a standard, 1- α -lysophosphatidyl-inositol in DMSO- d_6 [*l*-acyl-2-lyso-*sn*-glycero-3-phospho-(1-*D*-*myo*-inositol), $\delta_{\text{H-1/H-1'}}$ 3.99/3.97, $\delta_{\text{H-2}}$ 3.75, $\delta_{\text{H-3/H-3'}}$ 3.77/3.75; 1-lyso-2-acyl-*sn*-glycero-3-phospho-(1-*D*-*myo*-inositol), $\delta_{\text{H-2}}$ 4.78]. δ 3.97 was attributed to P4 glycerol H-1/H-1' and δ 4.00 to P5

glycerol H-1/H-1' (Figure 3a, Table 2). The chemical shifts recorded for the glycerol protons of P4 and P5 were in complete agreement with those obtained for standards, demonstrating that P4 and P5 belonged to monoacylphosphoglycerol units and more specifically to 1-acyl-2-lyso-*sn*-3-phospho-glycerol units. Taken together, these data demonstrated that, in common with P1 and P3, the PI anchor containing P4 and P5 phosphates was glycosylated on *myo*-inositol C-2 and C-6. *myo*-Inositol was acylated on C-3 in P4. In both PI units the glycerol was only acylated on C-1.

The resolution of the 1D ^1H spectrum obtained in DMSO- $^2\text{H}_6$ (Figure 4b) was considerably better than that obtained in $^2\text{H}_2\text{O}$ (Figure 4a). Weak resonances could thus be attributed by 2D ^1H - ^{31}P experiments, such as the H-3 of *myo*-inositol in the PI anchor characterized by P1 and the glycerol H-1 in the PI anchors characterized by P1 and P3 (Figure 4c). The anomeric resonances were assigned from the ^1H - ^{13}C -HMQC, -HMQC-HOHAHA and ^1H - ^1H -HOHAHA experiments (results not shown).

DISCUSSION

ManLAMs are amphipathic 17 kDa macromolecules characterized by a hydrophobic PI anchor and a hydrophilic carbohydrate domain. This asymmetry in polarity leads to the formation of aggregates. The motion of ManLAMs in solution thus tends to be much slower than expected from the molecular mass of a single molecule. This means that the overall rotational correlation times, τ_c , are much larger than expected, and the NMR transverse relaxation time T_2 tends to be small, leading to line broadening [34]. For example, the 1D ^{31}P spectrum of cellular ManLAM recorded in $^2\text{H}_2\text{O}$ exhibits a broad unresolved signal with a width of 150 Hz.

In order to improve the resolution of the 1D ^{31}P -NMR spectra of ManLAMs, we tested two different solubilization procedures. ManLAMs were first tentatively dissolved in water with the aid of detergents. Based on the method of purification by Triton X-114 phase separation [11], ManLAMs were initially solubilized in Triton X-114 and Triton X-100 [36]. Unfortunately, no ^{31}P signals were obtained in 1D experiments. We then tested DOC, which has been found to solubilize phospholipids in water and to improve the resolution of 1D ^{31}P -NMR spectra [37,38]. Two well-separated fine ^{31}P resonances were observed, but 2D data were not adequate for structural analysis. We hoped that an increase in the detergent-to-ManLAMs ratio would improve the 1D ^{31}P spectra [35,39], but the resulting mixture with 2% DOC was a gel at room temperature and was unsuitable for NMR analysis.

The first approach being unsuccessful, we then tested DMF- $^2\text{H}_7$, which has been reported to be a good solvent for ^{31}P -NMR analysis of phospholipids [33]. The 1D ^{31}P spectrum of ManLAMs solubilized in DMF/water exhibited an intense fine ^{31}P resonance but since the two signals evidenced using DOC were superimposed, we resorted to DMSO- $^2\text{H}_6$. Despite a lower resolution than that obtained in 1% DOC/100 mM EDTA, the separated resonances provided well-defined correlations in 2D experiments. In contrast to the experiments carried out with DOC, temperature was shown to have a marked influence. At 303 K, P1 and P3 resonate as a broad unresolved signal, while at 323 K the two signals were well separated with an increase in the signal-to-noise ratio at 343 K. DMSO- $^2\text{H}_6$ thus appeared to be an efficient solvent system for ^{31}P -NMR analysis of ManLAMs, enabling separation of phosphate resonances poorly resolved in $^2\text{H}_2\text{O}$ (9–16 Hz linewidth in DMSO- $^2\text{H}_6$ versus 150–200 Hz in $^2\text{H}_2\text{O}$). The phosphates detected belong to PI anchor units differing in structure and particularly in number of fatty acid

substituents. Indeed, $\delta^{31}\text{P}$ decreased with increase in the number of fatty acids esterifying the anchor. The number of fatty acids on the glycerol was shown to have a greater effect on the ^{31}P chemical shift than did the fatty acids on the *myo*-inositol. For example, the presence of a second fatty acid on the glycerol led to an upfield shift of 1.7 ppm, whereas on the *myo*-inositol a fatty acid only shifted the ^{31}P signal upfield by 0.17 ppm. DMSO- $^2\text{H}_6$ is convenient as it is liquid at room temperature, and both DMSO- $^2\text{H}_6$ and TEA are readily removed under a stream of nitrogen in contrast to detergents and salts, which require further purification by dialysis, partition or precipitation. This solvent system can be extended to the structural analysis by ^1H -NMR of other lipoglycans. For instance, we were able to visualize, on the 1D ^1H spectrum (Figure 4), the deshielded *myo*-inositol H-3 resonance at δ 4.59, which represents less than one proton out of a total of around 1200 (if we consider that ManLAMs contain approximately 600 protons).

The range of ^{31}P $\nu_{1/2}$ (150–200 Hz in water, 9–16 Hz in detergents with water or in organic solvents) enabled study of the aggregation pattern of the ManLAMs. In water, the observed signals were quite broad, indicative of a restricted motion of the phosphate groups probably due to high-molecular-mass aggregation arising from hydrophobic interactions between the fatty acyl chains of the ManLAMs. In contrast, the existence of small micelles (in water with DOC) or inverse micelles (in DMF and DMSO) allow a high degree of molecular motion and the observed signals are consequently sharp. These findings are also consistent with ^1H experiments. In DMSO, as opposed to water, the resonances corresponding to the protons at positions 1 and 2 of glycerol or the H-3 of the *myo*-inositol units are quite perceptible in 1D ^1H spectra (Figure 4).

Structural studies of ManLAMs have mainly been conducted using alkali-treated molecules due to their amphipathic nature and the difficulties encountered for their solubilization. We report here the first structural study using a non-destructive method revealing the anchor heterogeneity and allowing the determination of the number and localization of fatty acids in native multiacylated ManLAMs.

With respect to the biosynthetic pathway of ManLAMs, it is noteworthy that the 1D ^{31}P spectrum of cellular LMs from *M. bovis* BCG recorded under the same conditions as those described here exhibited exactly the same profile (resonances at the same chemical shift with the same intensities). These results, indicating that LMs contain the same PI unit forms as ManLAMs in the same ratio, further support a biosynthetic link between LMs and ManLAMs, recently suggested by Besra et al. [23].

The heterogeneity concerning the acylation degree was advanced by Leopold and Fischer [17] showing that *M. tuberculosis* ManLAMs are esterified by 2 or 3 fatty acids. In a previous study, we found that the PI unit of cellular ManLAMs of *M. bovis* BCG exhibited a large heterogeneity due to a combination of mono- and diacylglycerols [11]. The major form of diacylglycerols was 1-tuberculostearoyl-2-palmitoyl-*sn*-glycerol, whereas the major forms of monoacylglycerols were 1- or 2-palmitoyl-*sn*-glycerol. Nevertheless, monoacylglycerols were less abundant and represented only 27% of the acylglycerol forms as determined by GC/MS quantification. In the present study, we found only 13% of monoacylglycerols by integration of the ^{31}P -NMR signals (P4+P5). This latter value is probably more accurate since it is based on a non-destructive approach. Our study [11] and that from Brennan's group [22] allowed the localization of 3 acylation sites [22], i.e. positions 1 and 2 of glycerol and 6 of the Manp linked to C-2 of *myo*-inositol. We demonstrate here the presence of a fourth acylation site localized at position 3 of *myo*-inositol. We have found that this position in

Figure 5 Partial structural models proposed for the ManLAMs characterized by P1, P3, P4 and P5 phosphates

The PI anchor structure is detailed. The acylation state of position 6 of Manp linked to C-2 of *myo*-inositol was not established in this study, but this position is acylated in the fourth model since it is a potential acylation site [22]. The nature of the fatty acids on glycerol is in agreement with our data on the ManLAM acylglycerol structure [11]; only the major forms are represented but other combinations are possible. Manp are esterified by a palmitic acid in agreement with Khoo et al. [22]. The nature of *myo*-inositol acyl substituents remains unknown. P1 ManLAMs represent approximately 47%; P3 ManLAMs, 40%; P4 ManLAMs, 6%; and P5 ManLAMs, 7%. A corresponds to the carbohydrate moiety of ManLAMs.

the LMs and PIMs may also be acylated (M. Gilleron, J. Nigou, B. Cahuzac and G. Puzo, unpublished work). Interestingly, C-3 of *myo*-inositol has also been demonstrated as a potential acylation site of glycosylphosphatidylinositol-anchored proteins [40].

The degrees of acylation of glycerol and *myo*-inositol units were deduced from the 2D ^1H - ^{31}P -HMQC-HOHAHA experiment. Unfortunately, our analytical approach did not allow the determination of the acylation state of the Manp units. ^1H - ^{13}C -HMQC analysis designed to visualize correlations between fatty acid carbonyls and Manp H-6 was unsuccessful due to the low sensitivity of this experiment. In summary, from the present study, it can be advanced that P1 ManLAMs contain at least 3 fatty acids (2 on glycerol, 1 on *myo*-inositol); P3 ManLAMs, 2 (both on glycerol); P4 ManLAMs, 2 (1 on C-1 of glycerol, 1 on *myo*-inositol); and P5 ManLAMs, 1 (on C-1 of glycerol) (Figure 5). Moreover, a fatty acid has been described on the C-6 of an α -Manp unit [22], so we envisaged a highly acylated form of

ManLAMs, i.e. tetra-acylated, namely Ac_4ManLAM . This structure is supported by the existence of Ac_4PIM_2 , the form we unambiguously established for PIM_2 (M. Gilleron, J. Nigou, B. Cahuzac and G. Puzo, unpublished work). *M. bovis* BCG cellular ManLAMs exist in different forms depending on the number of fatty acid substituents ($\text{Ac}_n\text{ManLAMs}$, $n = 1-4$). ManLAMs with diacylglycerol forms represent approximately 87% (integration of P1+P2+P3 signals) of the total amount of ManLAMs, 47% (P1) being acylated on the C-3 of the *myo*-inositol. The remaining 13% (P4+P5) of ManLAMs contain 1-acetyl-2-lysoglycerol units, 6% (P4) being acylated on the C-3 of the *myo*-inositol (Figure 5).

The biological studies to date have been conducted on these ManLAM complex mixtures, typified by anchor heterogeneity. The PI anchor is known to be a crucial structural feature of ManLAM, since deacylation abrogates all the observed biological activities [6,7,11,19]. It is intriguing to speculate that these different acylated forms of ManLAMs could have quite different

activities. Indeed, we found that the structurally different parietal and cellular ManLAM PI anchors differentially stimulate interleukin-8 and tumour necrosis factor- α secretion from human dendritic cells [11]. Moreover it is likely that PI anchor plays a crucial role in ManLAM presentation to $\alpha\beta$ T cells by CD1 molecules [41]. CD1 glycoproteins are characterized by a hydrophobic groove which corresponds to the antigen-binding site [20]. It is quite clear that ManLAM binding to CD1 occurs through the hydrophobic interactions between ManLAM fatty acids and non-polar amino acids of the CD1 groove [41]. So, subtle differences in the PI anchor concerning the degree of acylation (from 1 to 4 fatty acids), the acylation site (glycerol, Manp, myo-inositol) and the nature of the fatty acids [palmitic, tuberculostearic, stearic, 12-*O*-(methoxypropanoyl)-12-hydroxystearic acids], probably discriminate ManLAM CD1 binding. Indeed, a recent study designed to establish the structural requirements for glycolipid recognition by CD1b molecules demonstrated that 6-mycoloyl-D-glucopyranose recognition was dependent on the presence of α -branched chains and β -hydroxy groups that typify mycolic acid structure [42]. Likewise, we could envisage that two fatty acids esterifying the glycerol are required to mediate ManLAM binding to the CD1 groove, banishing ManLAM species with monoacylglycerols (Figure 5). But, the situation is more complex concerning the role of fatty acids on Manp and myo-inositol units. They could perhaps induce steric effects, preventing the access of ManLAMs bearing these acyl appendages (Figure 5) to the groove. This latter is only accessible through a narrow entrance 6–7 Å wide [20].

Our findings represent a first step in the understanding of the structural basis responsible for the ManLAM discrimination in the presentation process by CD1b observed by Sieling et al. [19]. These authors have demonstrated that T-cell recognition requires an intact PI unit. The central role played by the ManLAM PI anchor is highlighted by a recent study [21] demonstrating that glycosylphosphatidylinositol is the major natural ligand of mouse CD1d1, corresponding to human CD1d. The different acylated forms of cellular ManLAMs need now to be obtained in a pure state in order (i) to describe the structure of the PI anchor precisely, and particularly the distribution of the fatty acids, especially on the Manp unit, and (ii) to study the binding of the different acylated ManLAMs to CD1. Taken together the resulting data would throw more light on the molecular mechanisms of ManLAM antigen recognition by T cells. The analytical approach described here is now successfully extended to the comparative structural analysis of the ManLAM PI anchor from *M. tuberculosis* and *M. bovis*.

CD1-restricted T-cells contribute to the increase of protective immunity, particularly against intracellular pathogens such as *M. tuberculosis*, which possess envelopes abundantly composed of lipoglycans and glycolipids. The determination of the molecular basis responsible for ManLAM binding to CD1 could constitute an important step in the prediction of a glycolipidic vaccine targeted for non-polymorphic class CD1 molecules [43].

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