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Mycobacterial glycopeptidolipid interactions with membranes: a monolayer study

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Abstract Mycobacterial glycopeptidolipid (GPL) interactions with membranes were analysed with monolayer experiment, using GPLs bearing 3, 1, or 0 carbohydrate residues (GPL3, GPL1, GPL0). Compression isotherms and surface potential determinations suggested that the glycopeptidic moiety of GPL3 permanently dipped in water, while those of GPL1 and GPL0 can lay in the interface. Insertion of GPL molecules into a preformed phospholipid monolayer was observed using GPL3 or GPL1 dispersions, but not from GPL0. It is postulated that the activity of GPL0 is low due to its failure to become inserted into membranes, as is that of GPL3 owing to its insertion only by its acyl chain. GPL1 is likely to disturb membranes by inserting its glycopeptidic moiety into the interface.

Key words: Mycobacteria; Glycopeptidolipid; Air–water monolayer; Lipid–membrane interaction

1. Introduction

Mycobacterial species of the 'MAIS' taxonomic group (*Mycobacterium avium, intracellulare, scrofulaceum*) are opportunistic pathogens infecting immunodeficient patients. They are intracellular parasites, multiplying within the host's macrophages. Glycopeptidolipids (GPLs) are present in their bacterial cell wall. These molecules share the same peptidolipidic backbone (Fig. 1) and the oligosaccharide non-reducing end of the molecule determines the serovar of the strain [1].

We have previously shown that aqueous dispersions of GPLs added to liposome suspensions drastically increased, without delay, the membrane permeability, as well as uncoupling oxidative phosphorylation when added to isolated mitochondria [2]. As GPLs are produced within infected macrophages [3], it can be postulated that they could help the pathogen to survive in the phagocyte by impairing membrane-linked functions of the host cell.

We took advantage of structural analysis that we had performed on GPLs differing by the number and the distribution of carbohydrate residues [4,5] to study the influence of the carbohydrate part of these molecules on their activity: the more polar compounds were the least active [6]. This was not due to differences in diffusion velocity of GPLs towards the membranes, since assays on mitochondria have shown that a preincubation step did not significantly increase the activity of the GPLs (unpublished data). Thus, the differences in activity of the three GPLs could result from differences in the interactions between the glycopeptidic moiety and membranes, especially with the phospholipidic part of membranes, since the same scale of activity for different GPLs has been obtained with mitochondria and with liposomes [6].

To study the phase behaviour and the interaction of GPLs with phospholipids, monolayers at the air-water interface were used as model membranes. Compression isotherms of pure GPL monolayers and the corresponding surface potential data were recorded. We also compared the insertion of GPLs of different polarities into phospholipid monolayers, since it has been shown, for free fatty acids, that there is a relationship between their interfacial properties and some of their effects on biological membranes [7].

2. Materials and methods

The GPLs used (Fig. 1) were prepared from *Mycobacterium peregrinum* (GPL3, 1300 Da) and *M. smegmatis* (GPL2, 1100 Da), as previously described [4,8]. The lipopeptide (GPL0, 870 Da) was isolated from *M. avium* serovar 2 (strain 2151, mutant Rg1) [9].

GPL1 (1000 Da) was prepared from GPL2 by removing the sugar residue linked to *allo*-threonine by alkaline β-elimination [4].

M. avium serovar 2 was grown on liquid Middlebrook 7H9 enriched medium, at 30°C for one month. Pelleted cells were harvested after centrifugation at 8,000 rpm for 30 min. Lipids were first extracted in chloroform/methanol (1:2, v/v) for a few days, then in chloroform/methanol (2:1, v/v). Pooled extracts were concentrated, washed with water, and evaporated to dryness.

Crude lipids were separated on Florisil columns (60–100 mesh) with increasing concentrations of methanol in chloroform. Fractionation was monitored by thin-layer chromatography on silica-coated plates (silicagel G60, Merck, 0.2 mm thickness) developed with chloroform/methanol (12:1, v/v) three times. Sugar-containing compounds were visualised by spraying plates with 0.2% anthrone in concentrated sulfuric acid, followed by heating. GPL0 (named LP-I and LP-II in ref. 9) were eluted by 5% methanol in chloroform. The presence of the lipopeptide was checked by infrared spectroscopy.

To prepare suspensions in water, GPLs were dissolved in hexamethylphosphoramide (HMPA). This solution was progressively added, under vortexing, to a 5 mM sodium phosphate, 45 mM NaCl buffer, pH 7.2. The temperature was maintained close to 40°C. The final concentration was $1 \cdot 10^{18}$ molecules/ml for GPL0 and GPL1, and $1 \cdot 10^{19}$ molecules/ml for GPL3.

Monolayer studies were carried out with divices made in the laboratory [10]. The surface pressure measured with the Wilhelmy platinum plate method and surface potentials were determined using two americium electrodes; experimental procedures have been previously described [10].

Lipids were spread from chloroform/methanol (7:3, v/v) solutions and the solvent was allowed to evaporate for 5 min before compression of the monolayer. Experiments were performed at 20°C on a 5 mM potassium phosphate, 45 mM NaCl subphase, pH 7.2. All the surface pressure-area isotherms presented here are the average of at least three measurements; they were reproducible to within 0.02 nm^2/molecule.

For insertion experiments, a Teflon trough with a 10 ml subphase volume was used. The GPL suspension (100 μl) was added to the subphase with a syringe through a side hole; changes in the surface pressure were measured with time using the platinum plate method.

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Addition in the water phase of amounts a volume of HMPA equivalent to that used to prepare GPL suspensions modified the surface tension of water (ca 4 mN/m); this value was substracted from the pressures recorded experimentally.

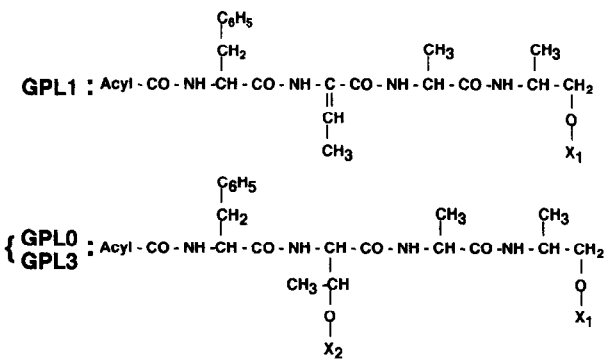
3 Results and discussion

3.1. Compression isotherms of GPL monolayers

GPL1 and GPL3 showed monotonous compression isotherms (Fig. 2). A film collapse was observed above a surface pressure of 30 mN/m, corresponding to limiting molecular areas of 0.70 and 1.35 nm^2 for GPL1 and GPL3, respectively. No film collapse was observed with GPL0.

From the comparison of these curves it can be concluded that: (i) the sugar moieties largely contribute to the molecular area of GPLs in monolayers; (ii) GPL1 displayed a more expanded compression isotherm than GPL0 and GPL3 since increasing the surface pressure from 5 mN/m to 30 mN/m led to a decrease in the molecular area of 1.4 nm^2 for GPL1, compared to decreases of 0.8 and 0.9 nm^2 for GPL0 and GPL3 respectively. GPL1 is a more compressible molecule than GPL0 and GPL3, and thus it is likely that this molecule is able to adopt a larger range of conformations and orientations at the interface than the other two GPLs.

The shape of the compression isotherms for the aglycon molecule (GPL0) is quite different from those of the corresponding glycosylated molecules, suggesting that the sugar



	GPL0	GPL1	GPL3
X_1 :	H	3,4 - di - O - Me rhamnosyl or 2,3,4 - tri - O - Me rhammosyl	3,4 - di - O - Me rhamnosyl (1 -> 2) 3,4 - di - O - Me rhamnosyl
X_2 :	H	-	3 - O -Me rhamnosyl

Acyl :				
	GPL0	C_nH_{2n-1} - CH - CH_2	n : 29,31	R : H
	GPL1	C_nH_{2n-1} - CH - CH_2	n : 25,27	R : CH_3 or H
	GPL3	C_nH_{2n+1} - CH - CH_2	n : 23,25	R : CH_3 or H

Fig. 1. Chemical structure of the tested glycopeptidolipids and lipopeptide. The number following GPL corresponds to the number of carbohydrate residues linked to the peptide moiety.

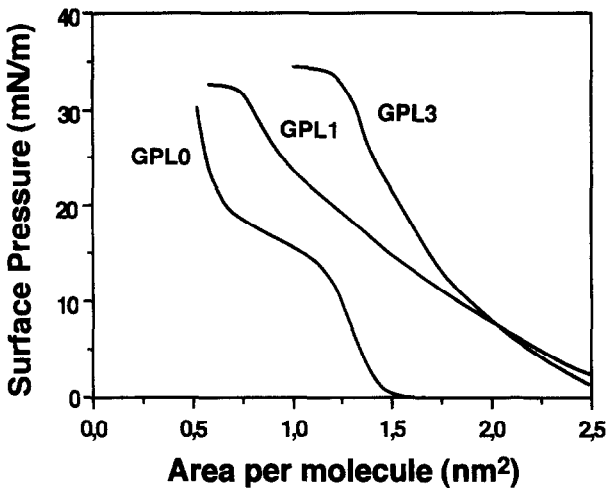


Fig. 2. Surface pressure/area isotherms of GPLs.

moieties of GPLs have an influence on the conformation and orientation of the peptide at the air-water interface. The central part of the GPL0 compression isotherm presents a quasi-plateau between 13 and 20 mN/m which accounts for a large change in molecular area for a small change in surface pressure. This can be interpreted as a transition of the molecule between two states, corresponding either to different ordering parameters for the acyl chains, or to different orientations of the peptide moiety.

It is well known that in case of phase transition from a liquid-expanded to a liquid-condensed state in monolayer, the transition plateau which is observed in the corresponding compression isotherm occurs at higher temperatures and with a decreased width as temperature is increased. Therefore, and in order to discriminate between the two above possibilities, compression experiments were performed at various temperatures between 20 and 36°C. No change in the compression isotherm of GPL0 was observed, indicating that an acyl chain melting was not responsible for the observed transition plateau. Instead, this plateau can account for a conformation or orientation transition of the peptide moiety. This is in agreement with fluorescence polarisation data onbtained with GPL0 that showed that the suspension was in the same rigid state from 10°C and to at least 35°C (data not shown). It is thus very likely that a conformation or orientation transition of the peptide moiety accounts for the plateau in the GPL0 isotherm.

To address this question, the surface potential ΔV of monolayers of the three GPLs was measured along with the compression isotherms (Fig. 3). It should be remembered that for neutral molecules, ΔV corresponds to the polarization potential ΔV_p, which is proportional to the projection of the dipole moments of the molecule (including the associated water molecules) onto the normal to the monolayer. Because of the large dipole moments of the peptide bonds, changes in conformation or orientation of the polar head of GPLs may result in large changes in ΔV. Fig. 3 shows the measured ΔV values and the $\Delta V/n$ values which correct for changes in the surface concentration of molecules resulting from film compression.

Comparison of Fig. 3A,B and C indicates different behaviors for the three lipids. For GPL0, both ΔV and $\Delta V/n$ abruptly decreased for molecular areas below 1.1 nm^2/molecule (Fig. 3A), indicating an important modification of the conformation

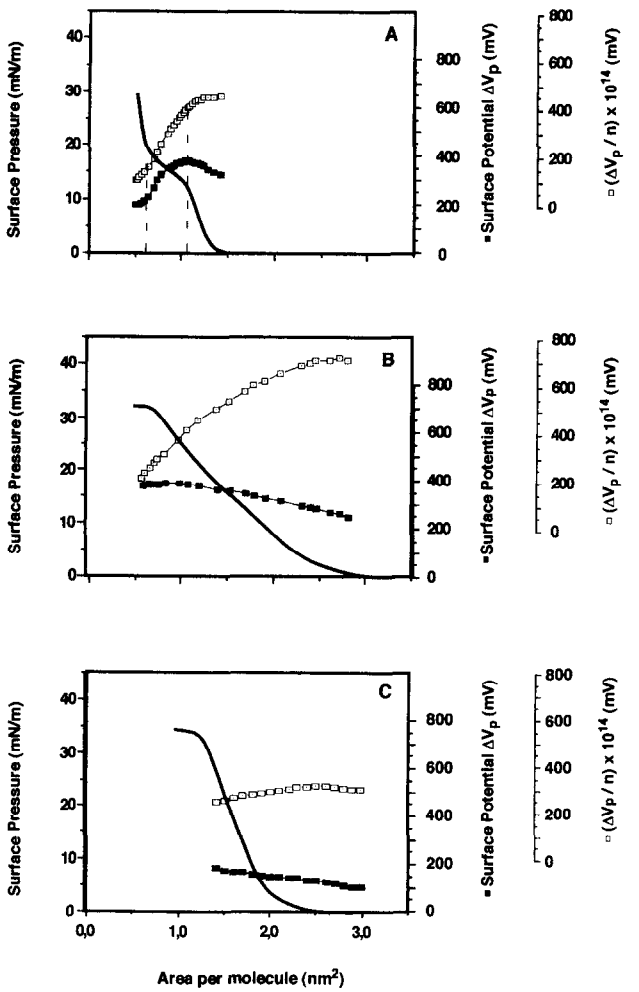


Fig. 3. Changes in surface potential (ΔV) upon film compression. Full line: compression isotherm. Full squares: ΔV_p experimentally measured; open squares: values corrected for changes in molecular area upon film compression ($\Delta V_p/n$). 5A, GPL0; 5B, GPL1; 5C, GPL3.

or of the orientation of the molecule at the interface. In contrast, ΔV and $\Delta V/n$ remainded practically constant for GPL3 (Fig. 3C), suggesting that no significant change occurred in the conformation or orientation of this lipid upon film compression. Curves corresponding to GPL1 (Fig. 3B) presented an intermediate situation: ΔV slightly increased upon film compression while $\Delta V/n$ strongly decreased during compression. It is thus likely that the conformation or the orientation of the GPL1 molecules changed.

The observed variations of surface potential do not provide direct information on the organisation of molecules in the film. However, in addition to the compression isotherm data, they show that the organisation of GPL0 and GPL1 molecules in the monolayer was greatly affected by film compression. This can be interpreted by transposing to monolayers the hypothesis we formulated previously for GPL/membrane interaction [6]: the glycopeptidic moiety could either lay at the air/water interface for low surface pressures, or dip into the water phase for high surface pressures. GPL0 and GPL1 would move from one conformation to the other upon film compression, may be in a cooperative way in case of GPL0 since a transition plateau was observed in its isotherm. On the contrary, both Π and $\Delta\Pi$ suggest that GPL3 kept a nearly constant conformation or orientation, with its very hydrophilic glycopeptidic moiety dipping in the water phase.

3.2. GPL dispersions and monolayers

To act on membranes, GPL molecules have to diffuse from the bacteria within the host cells in the course of an infection, or from suspensions in the assays we performed previously [2,6]. The ability of GPL dispersions to interact with membranes was tested by analysing the way these molecules, when dispersed in water, can form an monolayer at the air-water interface, or can interact with a preformed phosphatidylcholine monolayer.

As seen in Fig. 4, a monolayer was rapidly formed after injection of GPL dispersions in water, as detected by an increase in surface pressure. Maximum surface pressure was reached after about 15 min, close to 5, 23 and 32 mN/m for GPL0, GPL1 and GPL3 respectively. In order to compare this behaviour to that of phospholipids, egg-phosphatidylcholine liposomes (MLV) were tested: they formed a monolayer with a maximum surface pressure of 2 mN/m reached after 20 min.

GPL dispersions were also injected in the water phase below preformed egg-phosphatidylcholine monolayers, at various initial surface pressures (Π_i). Fig. 5A shows the result of experiments performed with an initial surface pressure of 10 mN/m. GPL1 and GPL3 dispersions induced a rather large Π increase, that reached a maximum within 5 min. A very Π small increase was observed with GPL0 suspensions.

From these results it can be concluded that GPL1 and GPL3 molecules are able to become inserted within the phospholipid monolayer, since they induced a large Π increase. GPL0 does not display the same behavior. It is worth noting that the insertion kinetics of GPL1 and GPL3 were very similar in spite of the difference in their sugar contents. This is in agreement with assays that we performed with mitochondria: a preincubation step did not significantly increased GPL activity (data not shown), indicating that differences in activity resulted from differences in the type of GPL/membrane interactions, but not from kinetic differences.

This kind of experiment was repeated for different initial

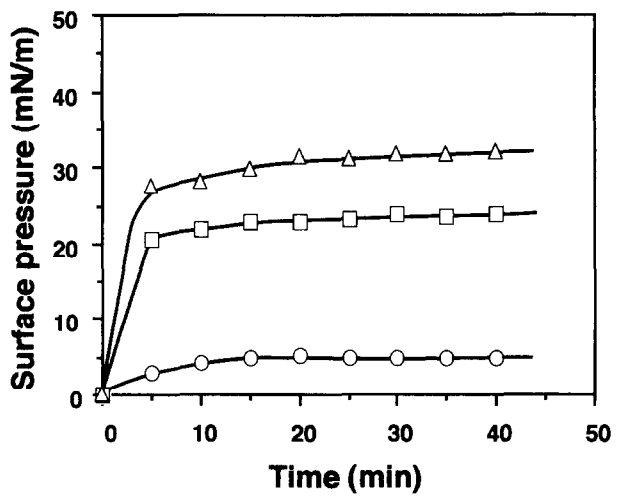


Fig. 4. Surface pressure of monolayers formed after injection of GPL suspensions in the water phase at constant surface area. Circles, GPL0; squares, GPL1; triangles, GPL3.

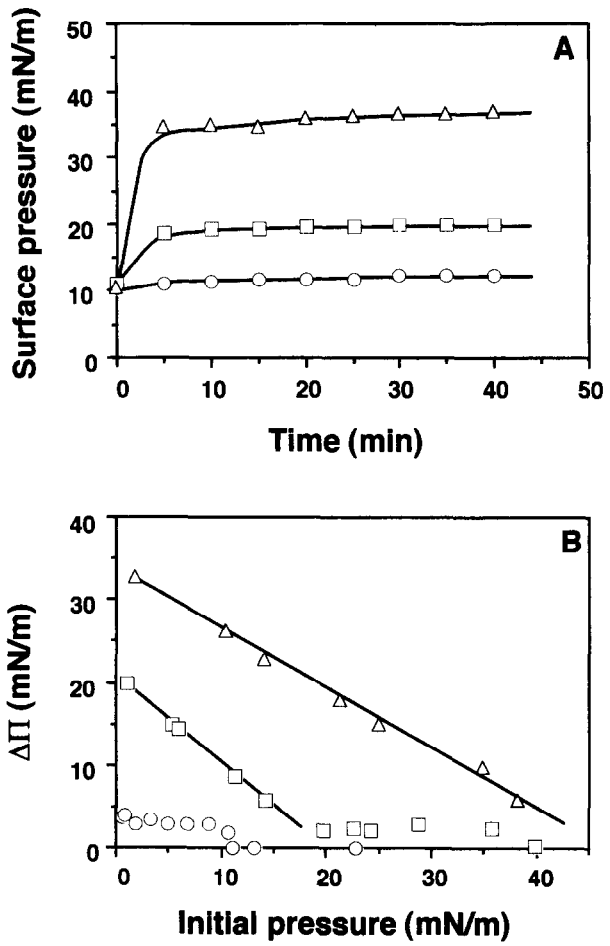


Fig. 5. Changes in surface pressure of preformed phosphatidylcholine monolayers after addition of GPL suspensions in the subphase. Circles, GPL0; squares, GPL1; triangles, GPL3. 5A, changes of Π with time, for an initial surface pressure Π_i of 10 mN/m; 5B, maximum values of $\Delta\Pi$, versus the initial surface pressure Π_i of the preformed phosphatidylcholine monolayer.

surface pressures Π_i of the egg-phosphatidylcholine monolayer and Fig. 5B shows the changes in surface pressure ($\Delta\Pi$) versus Π_i. For both GPL1 and GPL3, there was a linear decrease of $\Delta\Pi$, as Π_i was increased. The highest initial pressure permitting GPL insertion within the phospholipid layer can be estimated by extrapolating the curve down to $\Delta\Pi = 0$. An initial pressure limit of 20 mN/m was obtained for GPL1. A value of around 40 mN/m can be postulated for GPL3, a figure that was not checked experimentally because egg-phosphatidylcholine monolayers were not stable at this pressure in our experimental conditions. However, these data clearly indicate that GPL3 can be inserted within a monolayer more efficiently than GPL1.

For Π_i above 20 mN/m, GPL1 suspensions gave a small $\Delta\Pi$, not significantly dependent on the Π_i value used, up to $\Pi_i = 35$ mN/m. This could correspond to an adsorption of GPL1 molecules or particles to the monolayer surface, without insertion into the lipid layer.

GPL0 induced a small but significant $\Delta\Pi$ independent of Π_i, up to an initial pressure Π_i of 10 mN/m. This result is quite similar to that obtained by adding GPL1 to preformed monolayers with Π_i values above 20 mN/m, suggesting that GPL0 could interact with the phospholipid layer by a mere adsorption either of monomers or of the dispersed GPL particles.

It was estimated that in an egg-phosphatidylcholine monolayer prepared at $\Pi_i = 20$ mN/m, the number of GPL3 molecules inserted into the phospholipid layer was approximately ten times the number of GPL1 molecules inserted in the same conditions (data not shown).

Fig. 5B permits to compare the maximum Π observed for a monolayer formed from a dispersion ($\Delta\Pi$ for $\Pi_i = 0$), to the maximum Π resulting from insertion of GPL1 or GPL3 into a preformed phospholipid monolayer (Π for $\Delta\Pi = 4$). The figures obtained were close for GPL1 (20 mN/m versus 18 mN/m) and GPL3 (34 mN/m versus 42 mN/m). Thus, it appears that there is some relationship between the ability of a GPL suspension to modify the surface tension at the air/water interface and its ability to be inserted into a phospholipid monolayer: it seems that the upper limit in surface pressure permitting insertion of GPL from a suspension into a phospholipid layer is close to the surface pressure of the monolayer created by the same GPL suspension.

If so, it is not surprising to observe the near absence of insertion of GPL0 into a preformed monolayer at $\Pi_i = 10$ mN/m, since equilibrium between the lipid dispersion and the monolayer was reached for a surface pressure of 5 mN/m.

To conclude, the above data obtained with model membranes can help to understand the differences in the activity of GPLs on mitochondria. It should be remembered that GPL1 was far more active than GPL3 and GPL0 ([6] and unpublished data).

It is likely that the low activity of GPL0 resulted from its inability to become inserted into membranes, as it is shown here with phospholipid monolayers.

The large difference in activity observed between GPL1 and GPL3 is difficult to understand, but a few points can be considered: (i) the above data on GPL insertion into preformed phospholipid monolayers suggest that it was neither due to differences in the kinetics of interaction, nor to the number of inserted molecules; (ii) the above data obtained from GPL monolayers suggest that the bulky and hydrophilic GPL3 glycopeptidic moiety dipped in the water, with only its acyl chain in the membrane; if so in a natural membrane, this would not render GPL3 very disturbing; (iii) GPL1 had a compression isotherm presenting an extension on the abscissa nearly twice that of GPL0 and GPL3, suggesting a rather flexible molecule with several possible orientations. It is postulated that it can adopt two main orientations, with the glycopeptidic moiety either dipping in the water phase, or laying within the interface. This latter orientation could disturb the interactions between membrane constituents and also water organization at the interface, resulting in alteration of membrane functions.

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