Interactions of Mycobacterial Glycopeptidolipids with Membranes: Influence of Carbohydrate on Induced Alterations†

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ABSTRACT: Glycopeptidolipids (GPLs) are specific constituents of mycobacteria known as opportunistic pathogens. The influence of the carbohydrate moiety on GPL-induced membrane alterations was examined with GPLs bearing 1–5 sugar residues (GPL-1 to GPL-5) and a sulfated GPL (S-GPL-2). GPLs decreased the ADP/O ratio and increased controlled respiration of isolated mitochondria. The more polar GPLs were the less active, with the following order of efficiency: GPL-1 > GPL-2 > S-GPL-2 = GPL-3 = GPL-5. GPL-1 and GPL-2 increased passive permeability of liposomes to carboxyfluorescein (GPL-1 > GPL-2), while GPL-3 and GPL-5 were inactive. GPL-2 and GPL-3 decreased the transmembrane electrical potential ($\Delta\Psi$) in isolated mitochondria (GPL-2 > GPL-3). These results suggest that GPLs uncouple oxidative phosphorylation by increasing the passive permeability of the mitochondrial membrane to protons. Compression isotherms of GPL-2 monolayers showed that, at low surface pressure, the area per GPL-2 molecule was about 5 times that of an acyl chain: it is likely that the peptide moiety was at the air/water interface. With an increase in the surface pressure, its area decreased, down to that of a tightly packed acyl chain. It is postulated that the glycopeptidic moiety can be either at in the interface or dipping into the water. GPL-2 insertion in liposomes rendered the acyl-chain part of the bilayer more accessible to ions, since a fluorescent probe located deep in the bilayer was much more quenched by Cu^{2+} ions in liposomes containing GPL-2 than in control liposomes, suggesting a disturbance of the bilayer interface. A model is proposed to explain the influence of the polarity of GPLs on their activity toward membrane properties.

Pathogenic mycobacteria are intracellular parasites causing long-lasting diseases, the main ones being tuberculosis and leprosy. No molecular mediator of the pathogenic effects in the host is known, such as endo- or exotoxin, in contrast to most infectious diseases [for reviews, see Rastogi and David (1988), Draper (1989), Barrow (1991), and Lanéelle and Daffé (1991)]. Mycobacterial envelopes are very rich in lipids presenting structures quite different from those of common membrane lipids. It is currently postulated that these lipids could diffuse toward and become inserted into host membranes and impair their properties (Lanéelle & Tocanne, 1980; Woodbury & Barrow, 1989; Boddingius & Dijkman, 1989). Membrane disturbance could be one of the ways used by pathogenic mycobacteria to escape destruction in macrophages and to unbalance the delicate communication network in the cellular immune system, inducing indirect damages to the infected host (Shoenfeld & Isenberg, 1988). The possibility of participation of wall lipids in mycobacterial pathogenic effects was reinforced by studies on 6,6′-dimycoloyltrehalose (cord factor) which showed that this compound can be toxic for the mouse and that it interferes with the immune system [for reviews, see Lederer (1979) and Goren (1990)]. It has also been shown that phenol glycolipids suppress the oxidative response of monocytes (Vachula et al., 1989) and lymphocyte mitogenesis (Fournié et al., 1989) and that glycopeptidolipids (GPLs)[1] modify the lymphocyte response to mitogens (Hooper & Barrow, 1988; Tassel et al., 1992).

In a recent survey of the effects of some mycobacterial lipids on membranes (Sut et al., 1990), we showed that a glycopeptidolipid (also called mycoside C) present in several opportunistic pathogenic species of mycobacteria, such as *Mycobacterium avium* and *Mycobacterium chelonae*, permeabilizes liposome bilayers and disturbs oxidative phosphorylation in isolated mitochondria. GPLs contain from two to six carbohydrate residues, depending on the species or subspecies of *Mycobacterium* [for a review, see Brennan (1989)]. We took advantage of the discovery of novel GPLs (Lopez-Marin et al., 1991), including a sulfated one (Lopez-Marin et al., 1992), to study the structure–activity relationship of this family of molecules, and especially the influence of the carbohydrate moiety, and to define how these molecules are able to disturb membrane organization and function.

MATERIALS AND METHODS

Glycopeptidolipids (GPLs). The GPLs used in the present work (Figure 1) were prepared from *Mycobacterium peregrinum* (GPL-2, 1122 Da; S-GPL-2, 1214 Da; GPL-3, 1296 Da) as previously described (Lopez-Marin et al., 1991, 1992) or from *M. avium* serovar 4 (GPL-5, 1654 Da) (Mc Neil et al., 1988). Acetyl groups were removed from GPL-2 and GPL-5 by mild alkaline deacylation (Brennan & Goren, 1979). GPL-1 (956 Da) was prepared from GPL-2 by removing the sugar residue linked to the *allo*-threonine by an akaline

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[1] Abbreviations: 2-AP, 2-(9-anthroyloxy)palmitate; 12-AS, 12-(9-anthroyloxy)stearate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; DPH, diphenylhexatriene; GPL, glycopeptidolipid; GPL-*x*, glycopeptidolipid bearing *x* carbohydrate residues; S-GPL, sulfated GPL; SUV, small unilamellar vesicle.

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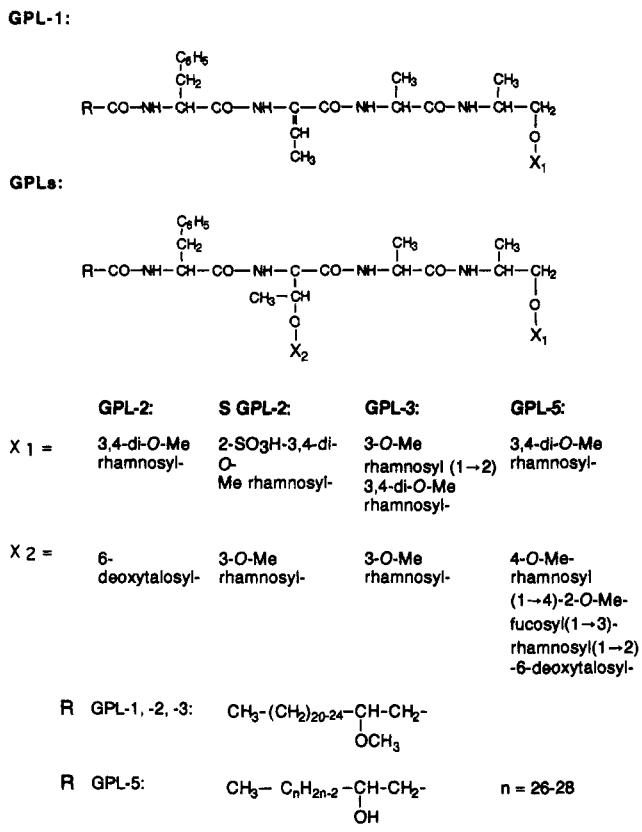

FIGURE 1: Chemical structures of the tested glycopeptidolipids (GPLs).

β-elimination reaction: pure GPL-2 was treated with 5% KOH in CH_3OH/C_6H_6 (8/2, v/v), at 70 °C for 4 h; the reaction product was isolated by preparative thin-layer chromatography.

Miscellaneous Compounds. The following compounds were purchased from Sigma: bovine brain phosphatidylserine, egg phosphatidylcholine and phosphatidic acid, diphenylhexatriene (DPH), 12-(9-anthroyloxy)stearate (12-AS), rhodamine-123, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and ADP. Dicarboxyfluorescein was purchased from Eastman and purified according to Weinstein et al. (1983). 2-(9-Anthroyloxy)palmitate (2-AP) was prepared acording to Lenard et al. (1974).

Glycopeptidolipid Suspensions. GPL (ca. 0.5 mg) was dissolved in 20 μL of hexamethylphosphoramide. Then 5 mM sodium phosphate, pH 7.2, was progressively added under vortexing, to obtain 1–2 mg of GPL/mL. The temperature was maintained close to 40 °C.

Assays on Mitochondria. Rat liver mitochondria were prepared and used for respiration and phosphorylation determinations according to routine laboratory procedures (Durand et al., 1979b). All preparations used had a respiratory control ratio of at least 3 (in the control experiments). GPLs were tested by direct addition of the glycolipid suspension to the assay cuvette. Active and controlled respiration states were obtained in the presence and absence of ADP, respectively.

Variations of the transmembrane electrical potential ($\Delta\Psi$) were followed by measuring the fluorescence intensity of rhodamine-123 (λ_{exc}, 500 nm; λ_{em}, 545 nm) according to Emaus et al. (1986). Briefly, rhodamine-123 was added (final concentration, 0.3 μM) to mitochondria (ca. 100 μg of protein/assay) suspended in the respiration medium. Ten minutes later, succinate was added (5 mM final concentration). After 3 min, the fluorescence increase had become stable and the GPL suspension was added; fluorescence decreased. Five minutes later, CCCP was added (0.1 μM final concentration), and fluorescence decreased to its starting value before succinate addition. The relative efficiency of GPLs was estimated by comparing the fluorescence intensity decrease induced by addition of 250 nmol of GPL to the total fluorescence decrease obtained after CCCP addition (taken as 100%).

Liposome Preparation and Use. The liposomes used for permeability assays were small unilamellar vesicles (SUVs). They were routinely prepared by sonicating (under argon) 4 μmol of a mixture of phosphatidylcholine, phosphatidylserine, and cholesterol (2/1/1, molar ratio) in 0.2 mL of 120 mM dicarboxyfluorescein in 5 mM Tris and 45 mM NaCl, pH 7.2. The liposomes were separated from the preparation medium on a Sephadex G-25 column (1 × 10 cm) and maintained at 4 °C until use. Leaks of dicarboxyfluorescein were followed by measuring the initial slope of the fluorescence increase with time (λ_{exc}, 492 nm; λ_{em}, 520 nm). The efficiency of the tested GPL was determined by calculating the ratios of the slopes of fluorescence intensity increase between the control and each assay.

The liposomes used for fluorescence quenching experiments were prepared with the same lipid composition and the same buffer as above. To test the effect of GPL-2, the liposomes also contained 10 mol % GPL-2. They were prepared by progressively injecting the lipid mixtures (dissolved in CH_2Cl_2) into the buffer at 50 °C, with vortexing. Fluorescent probes (2-AP or 12-AS) were added to the liposome preparations at a final concentration of 4×10^{-6} M. Nitrogen was bubbled through the preparation to eliminate the solvent.

Monolayers at the Air/Water Interface. Monolayers were prepared according to routine procedures with a laboratory-made apparatus (Sacré & Tocanne, 1977; Durand et al., 1979a). Surface pressure (Π) was measured with a Wilhelmy platinum plate for phosphatidylcholine–GPL mixtures, but the Langmuir barrier was preferred for monolayers of pure GPL since it gave more reproducible compression isotherms above 30 mN/m.

RESULTS AND DISCUSSION

Effects of Carbohydrate Residue Number on Oxidative Phosphorylation. Because rat liver mitochondria have to be used no later than 2–3 h after isolation, it was not possible to test all of the GPLs presented in Figure 1 on each mitochondrial preparation. So we compared all compounds to a reference GPL, the one with two carbohydrate residues (GPL-2).

Figure 2 illustrates the dose dependence of the effects of GPL-2, -3, and -5. It clearly appears that all of the compounds presented the same type of activity: an increase in controlled respiration correlated to a decrease of phosphorylation efficiency (ADP/O ratios). Active respiration was not significantly modified (not shown).

These effects look like those of protonophore classical uncouplers, and the efficiencies of the most active GPLs (GPL-1 and GPL-2) are close to that of a classical ionophore like dinitrophenol (DNP), since DNP completely uncouples isolated mitochondria when used at 10^{-4} M concentration, *i.e.*, 0.1 μmol/(mg of protein), and GPL-2 had the same effect at 0.3 μmol/(mg of protein).

The effects observed with GPLs are in contrast with that observed in the presence of 6,6′-dimycoloyltrehalose (cord factor), since cord factor also decreased the ADP/O ratio but inhibited active respiration (Durand et al., 1979). First, we chose to compare efficiencies by determining (from graphs

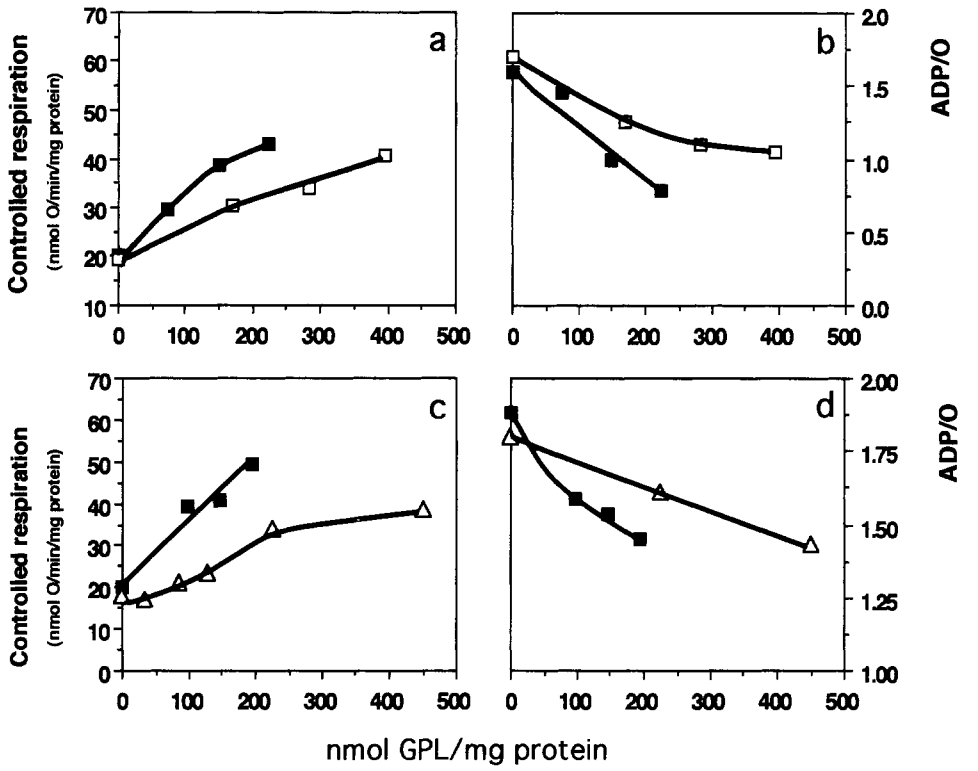

FIGURE 2: Effect of GPLs on ADP/O ratios and on controlled respiration rates in isolated rat liver mitochondria. GPL suspensions were added to mitochondria in the measurement cuvette before addition of ADP (succinate as substrate). GPL-2 was used as a reference compound in each assay series. (a, c) Controlled respiration rates [(nmol of O) min^{-1} (mg of protein)$^{-1}$]; (b, d) ADP/O ratios; (■) GPL-2; (□) GPL-3; (△) GPL-5.

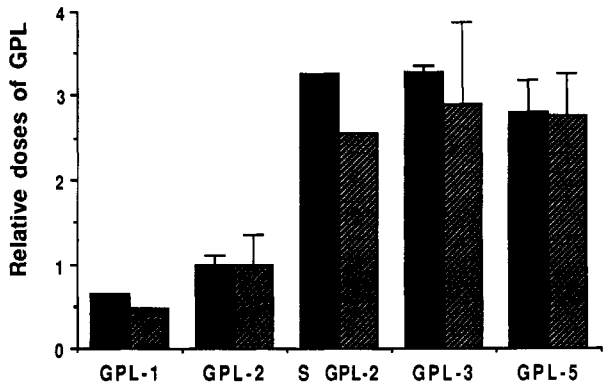

FIGURE 3: Comparative efficiencies of GPLs on ADP/O ratios and on controlled respiration rates. GPL amounts [nmol (mg of protein)$^{-1}$] needed to induce a decrease of 0.5(ADP/O) and the amounts inducing a 25% increase of controlled respiration were determined from graphs such as those in Figure 2. Then all the values were compared using the values obtained with GPL-2 as units. This gives a scale of the relative efficiencies of the GPLs. Solid bars, ADP/O; hatched bars, controlled respiration.

like those in Figure 2) the amounts of GPLs which had to be added to the mitochondria to decrease ADP/O by 0.5 and to increase controlled respiration by 25%. Then the values determined with GPL-2 were taken as unity (Figure 3). This approach allowed us to determine the GPL/GPL-2 ratios of the doses required to obtain the chosen effects, and thereby to assess the relative efficiencies of the GPLs.

It appears from Figure 3 that molecules with more than two carbohydrate residues (GPL-3 and -5) are much less active than the reference molecule GPL-2, while the molecule with only one residue (GPL-1) is slightly more active. The sulfated molecule (S-GPL-2) does not fit with this conclusion since, in spite of its sugar content (two sugar residues), it presented the same activity as GPL-3. But it must be considered that the sulfate group on the carbohydrate residue (X1 in Figure 1) renders S-GPL-2 at least as polar as GPL-3, in agreement with its chromatographic behavior (Lopez-Marin et al., 1992).

Inhibition of mitochondrial oxidative phosphorylation generally results from an alteration of the proton-motive force, due to an increase of the passive permeability to protons of the mitochondrial inner membrane. This is exemplified by the effect of currently used protonophores like CCCP. However, some molecules decreasing ADP/O do not significantly change the proton-motive force, for instance, long-chain fatty acids or gramicidin A (a quasi-ionophore peptide), and it has been proposed that this type of molecule does not induce proton leaks, but disturbs proton circulation in the membrane chemiosmotic systems (Rottenberg, 1990). As GPLs are not structurally related to the usual ionophores and are neither carboxylic acids nor peptides long enough to create a pore through a lipid bilayer, it was not possible to relate them to one of the two types of phosphorylation inhibitors without experimental data.

Carbohydrate Residues and Membrane Permeability. We have previously shown that GPL-2 induced leaks in liposomes containing dicarboxyfluorescein (Sut et al., 1990). The influence of the number of carbohydrate residues on this effect was examined.

Figure 4 shows the effect of 4 GPLs on liposome permeability to carboxyfluorescein. Two groups of molecules can be considered: GPL-1 and GPL-2 were clearly active, the former being roughly twice as active as the latter, while GPL-3 and GPL-5 did not show any activity with the chosen experimental conditions. These results are in agreement with the significantly higher activity on mitochondria observed with GPL-1 and GPL-2 compared to that of GPL-3 and -5. They suggest that GPL-induced uncoupling of oxidative phosphorylation could result from proton leaks through the inner membrane of the mitochondria.

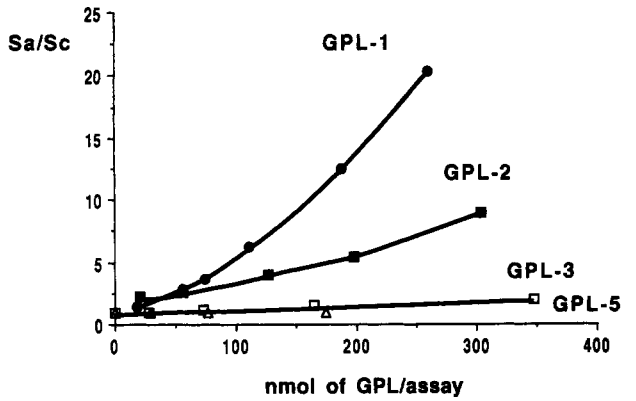

FIGURE 4: GPL effect on liposome permeability to carboxyfluorescein (CF). GPL suspensions were added to CF-containing liposomes. Sa and Sc are the slopes of the fluorescence increase with time (arbitrary units) due to CF leakage from assay (with GPL) or control (without GPL) liposomes, respectively.

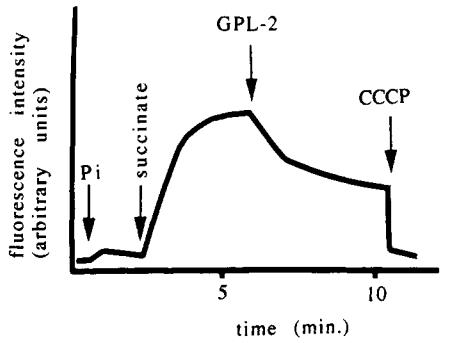

FIGURE 5: Effect of GPL-2 on mitochondrial transmembrane difference of potential ($\Delta\Psi$). $\Delta\Psi$ variations were followed by recording variations of rhodamine-123 fluorescence in mitochondria in the state of controlled respiration (succinate as substrate). At the end of each assay, $\Delta\Psi$ was abolished by addition of CCCP, and the total variation of fluorescence was taken as 100%.

As it is currently accepted that the transmembrane electrical potential ($\Delta\Psi$) is the major component of the proton-motive force in mitochondria [see Rottenberg (1990)], the effect of two GPLs on $\Delta\Psi$ was followed using the fluorescence of rhodamine-123, a $\Delta\Psi$-sensitive fluorophore (Emaus et al., 1986). As seen in Figure 5, addition of the substrate increased fluorescence, indicating the establishment of a $\Delta\Psi$, while fluorescence was decreased by GPL-2 and abolished by a powerful uncoupler (CCCP).

The effects of GPL-2 and -3 on $\Delta\Psi$ were compared by determining the decrease of fluorescence relative to the decrease induced by the presence of 10^{-5} M CCCP (taken as 100%). Addition of 250 nmol of GPL-2 to mitochondria in the controlled state of respiration decreased fluorescence by 47%, but only by 25% with GPL-3. Thus, GPL-2 reduced $\Delta\Psi$ roughly twice as efficiently as GPL-3. The same efficiency ratio (GPL-2/GPL-3) was noted by considering the effects of GPL-2 and -3 on the ADP/O ratios presented in Figure 2B, and as said above, GPL-2 was clearly more active than GPL-3 on liposome passive permeability. It is thus likely that the decrease of the ADP/O ratio in the presence of GPLs resulted from an alteration of the transmembrane electrical potential, due to proton leakage induced by GPLs.

How Could GPLs Disturb Membranes? Information on the possible arrangement of GPL-2 in a lipid layer was obtained from monolayers at an air/water interface. It can be seen from the compression isotherm of pure GPL-2 (Figure 6) that this molecule occupies about 1.5 nm^2 at this interface, with a lateral pressure of 20 mN/m. This is about 5 times the area

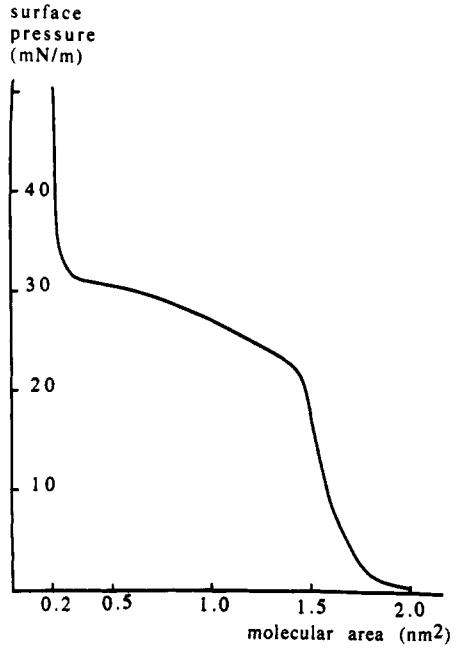

FIGURE 6: Compression isotherm of GPL-2 monolayers at the air/water interface.

needed, at this surface pressure, for the acyl chain of GPL-2 (ca. 0.3 nm^2) and corresponds roughly to the area occupied by two phospholipid molecules. Thus, it is reasonable to assume that at least part of the peptide moiety of GPL-2 was also present at the interface. Further compression of the monolayer resulted first in a gentle slope, up to 32 mN/m, and then in a nearly vertical curve corresponding to an area of about 0.2 nm^2, *i.e.*, that expected for a tightly packed acyl chain. This result can be interpreted as follows: at 20 mN/m, peptide moieties are packed close together at the interface, and further compression progressively pushes them into the aqueous phase. The same phenomenon was observed with monolayers containing phosphatidylcholine and GPL-2 (data not shown). This suggests that GPL-2 interacting with a membrane could have its acyl moiety inserted between the fatty acyl chains of the phospholipids, and its peptide moiety amid the polar heads, at the water/membrane interface. This should create a local and discrete defect in the membrane bilayer facilitating the penetration of water-soluble ions and molecules into the hydrophobic part of the membrane.

To test this hypothesis, use was made of liposomes labeled with the fluorescent probes 2-(9-anthroyloxy)palmitate (2-AP) and 12-(9-anthroyloxy)stearate (12-AS); the former has its fluorescent residue (anthracene) exposed to water, while the latter has the fluorescent residue located deep within the hydrophobic layer. Then the dynamic quenching of anthracene fluorescence by Cu^{2+} ions was examined on liposomes containing 10 mol % GPL-2 as compared to liposomes devoid of glycolipid (Figure 7).

It appeared that quenching of 2-AP was nearly as effective for liposomes with or without GPL-2 in the bilayer, while the presence of GPL-2 in liposome membrane strongly increased the quenching of 12-AS, rendering it nearly as intense as the quenching of 2-AP. So, the presence of GPL-2 inside the bilayer seems to render the anthracene moiety of 12-AS readily accessible to Cu^{2+}. With the defect created by GPL-2 making the hydrophobic part of the bilayer more accessible to water-soluble ions, the membrane becomes more permeable to ions, especially to protons.

Toward a Model To Explain the Influence of Carbohydrates on GPL Activities. It is proposed in the above section

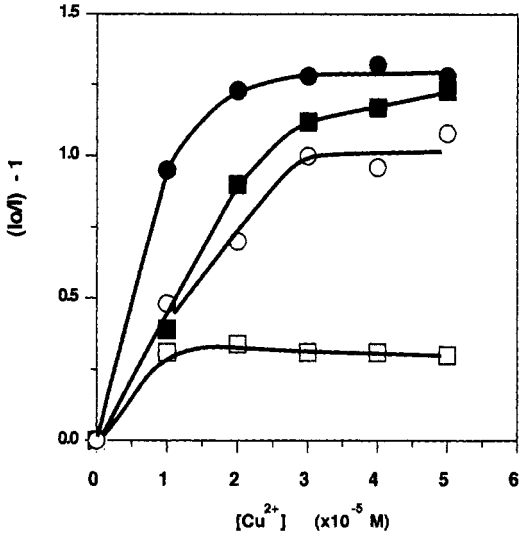

FIGURE 7: Effect of GPL-2 on dynamic quenching of 2-AP and 12-AS in liposomes. Liposomes prepared with or without 10% (molar ratio) GPL-2 were used to determine Cu^{2+} penetration in the bilayer by determining the quenching of two anthroyloxy acid probes. I and I_0: fluorescence intensity in the presence or in the absence, respectively, of GPL-2 in liposomes. (■, □) Liposomes with 12-AS; (●, ○) Liposomes with 2-AP. Solid symbols, assays (liposomes with GPL-2); open symbols, controls (liposomes without GPL-2).

that the peptide moiety of GPL-2 is inserted at the water/membrane interface at the level of the phospholipid polar heads. In contrast, it is likely that sugar residues are in the water phase, since it has been shown with mono- and diglycosyl diglycerides inserted into phospholipid bilayers that the sugar moieties were fully extended in water, away from the bilayer (Jarrel et al., 1987; Renou et al., 1989).

Compression isotherms of GPL-2 monolayers suggested that when the monolayer lateral pressure increased, the peptide moiety could be progressively expelled from the interface; thus, in the course of a compression isotherm, a fraction of GPL molecules has the glycopeptide moiety in the interface, while the remaining molecules have it in water. This suggested the following working model (Figure 8).

Once in the membrane, the GPL molecule has its acyl chain anchored within the lipid layer, while the glycopeptide moiety is engaged in a dynamic equilibrium between two locations, either adsorbed on the lipid layer or dipping into the water phase. The position of the equilibrium between these two locations of the glycopeptide moiety would depend both on the lateral pressure in the membrane and on the relative affinity of the glycopeptide moiety for water and for the interface. Affinities could depend on the hydrophobicity of the glycopeptide moieties of GPLs: an increase of the number of sugar residues should increase the partition toward water, but the nature of the sugars could also influence the affinity of the glycopeptide for the phospholipid/water interface.

The relative efficiency of the different GPLs tested is in agreement with this model since an increase in the polarity of the sugar moiety is likely to displace the equilibrium toward an increasing proportion of the glycopeptide moiety being out from the membrane interface: the least polar GPLs, GPL-1 and GPL-2, are considered to be located most of the time in the interface, while the most polar GPL, GPL-5, should be most often in the water phase.

A direct consequence of this model is that multiglycosylated GPLs, which are major serological determinants in *M. avium* and related species, should not be deleterious for host membranes since their glycopeptide moiety can be assumed

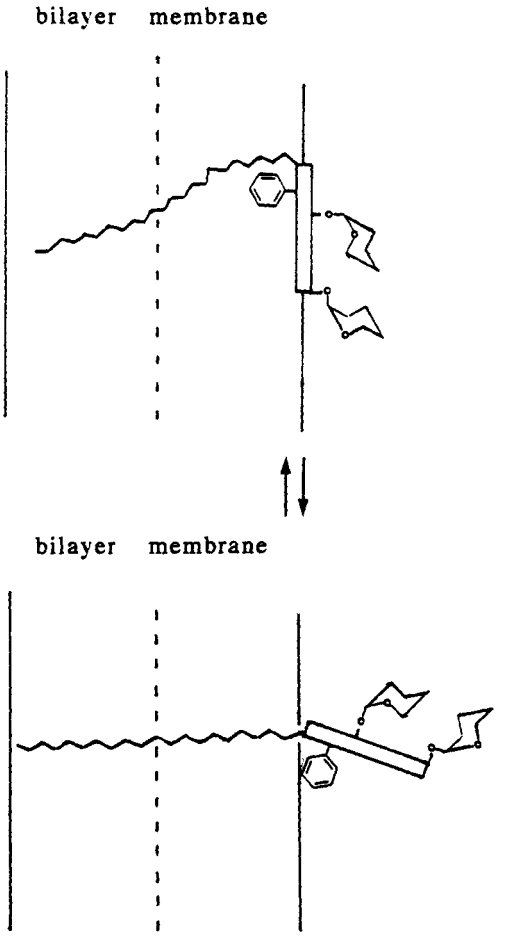

FIGURE 8: Model proposed for the insertion of GPLs in a bilayer membrane. The rectangle represents the peptide moiety. The GPL molecule is supposed to have its acyl moiety permanently inserted between phospholipid acyl chains. The peptide moiety is supposed to be engaged in a dynamic equilibrium between two positions: either inserted in the air/water interface of the membrane or dipping into the water phase. The distribution between these two positions should be imposed by the relative affinity of the glycopeptide moiety for water and phospholipids, respectively.

to be very poorly inserted into the membrane/water interface, but it could be presented by the competent cells to produce antigens since their carbohydrate epitopes are thought to be fully exposed in the water phase. In contrast, the so-called "apolar" GPLs (*e.g.*, GPL-2), generally present in GPL-producing mycobacterial species, are likely to have their glycopeptide moiety inserted in the interface, a position that increases membrane passive permeability. So, this should render them much more harmful for host cells and make them potential pathogenicity effectors of this group of opportunistic pathogens.

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