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Influence of monoglucosyldiacylglycerol and monoacylmonoglucosyldiacylglycerol on the lipid bilayer of the membrane from *Acholeplasma laidlawii* strain A-EF22

Alf R. Niemi, Leif Rilfors, Göran Lindblom *

Department of Physical Chemistry, Umeå University, S-90187 Umeå, Sweden

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Abstract

The ability for 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol (MGlcDAG) and 1,2-diacyl-3-*O*-(6-*O*-acyl-(α-D-glucopyranosyl))-*sn*-glycerol (MAMGlcDAG) to induce non-lamellar phases in a lipid mixture with an in vivo composition, prepared from *Acholeplasma laidlawii* membranes, has been investigated. The phase transition temperatures from lamellar to non-lamellar structures were studied with varying fractions of MGlcDAG and MAMGlcDAG. The transition temperature decreased from 73 ± 2°C for 20 mol% MGlcDAG to 43 ± 1°C for 63 mol% MGlcDAG, in lipid mixtures where the other lipids are the native bilayer-forming lipids. MAMGlcDAG behaved differently and the phase transition temperatures were found to be almost constant and between 51–53°C as the fraction of MAMGlcDAG varied between 11–45 mol%. It was also found that MAMGlcDAG can only be solubilized in low concentrations in the lipid bilayer, which is in good agreement with the fractions of MAMGlcDAG found in the membrane of *A. laidlawii*. Higher concentrations of MAMGlcDAG resulted in phase separations of lamellar liquid crystalline and gel/crystalline phases. It is concluded that MAMGlcDAG is far more capable than MGlcDAG to induce non-lamellar structures at lower concentrations. The results are discussed in terms of the model of lipid regulation previously proposed by this laboratory (Lindblom, G., Hauksson, J.B., Rilfors, L., Bergenståhl, B., Wieslander, Å. and Eriksson, P.-O. (1993) J. Biol. Chem. 268, 16198–16207), and the importance for the bilayer stability in cell membranes. It is proposed that the phase behaviour of the membrane lipids has far-reaching consequences for membrane function.

Keywords: Glucolipid; Phase equilibrium; NMR, ^{2}H-; Membrane; Regulation

1. Introduction

Lipids along with proteins are the major components of biomembranes. The structures that lipids form in the presence of water have provided the basis for the current models of biomembranes (see, e.g., [1–4]). The membrane lipids are in a bilayer arrangement characterized by the state of a lamellar liquid crystalline (L_α) phase. Because of the similarities in physico-chemical properties between a membrane and the L_α phase it is considered a relevant model for biomembranes. A study of membrane lipids, and the different liquid crystalline phases they form, is therefore of direct biological bearing.

The diverse functions of cell membranes demand the presence of a large number of proteins and lipids. Metabolically active membranes, like the inner mitochondrial membrane, often contain numerous enzymes and transport proteins. In like manner, diversity is found in the membrane lipids. Thus, the membranes of both eukaryotic and prokaryotic cells have lipids with different polar headgroups as well as a large variation in the length and unsaturation of the acyl chains. The number of different lipid species may be as large as several hundreds in a particular cell membrane. If the function of membrane lipids were only to provide a permeability barrier for cells, then a single lipid species such as dioleoylphosphatidylcholine would suffice, since it would maintain the bilayer properties necessary for a functioning membrane. Cells, however, go out of their way to regulate the components of their membranes and there must be some reason for the large number of different lipids found in a single cell membrane. Modulation of the lipid composition could affect both the structural and dynamic properties of the entire system [1,5–12]. It has been suggested that physico-chemical properties of the cell membrane such as

* Corresponding author. E-mail: goran.lindblom@physchem.umu.se. Fax: +46 90 167779.

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lipid bilayer thickness, interfacial area per lipid molecule, surface charge density, curvature stress or bending elasticity, may play an important role in the membrane functioning [4,11–13]. Therefore, it is of great importance to understand how different lipids, or other membrane components, influence these physical properties. Detailed investigations of the phase behaviour and phase structure of the principal lipid constituents (or even all of them) in a membrane most certainly help resolve many questions asked about, e.g., the membrane stability.

The ability of many membrane lipids to form non-lamellar phases in aqueous dispersions has received considerable attention over the past few years [5,14–20]. Most membranes contain at least one lipid species that in water forms non-lamellar phases. Phosphatidylethanolamines and monoglycosydiacylglycerols are the most common lipids that are able to form such phases. Although non-lamellar phases are assumed not to exist in biological membrane systems, the forces causing them to form are, however, present in the lipid bilayer and may play a role in membrane function [5,16,17]. It should be noted that the bilayer elasticity is determined by the lateral packing density and therefore a relation exists between the elasticity and the properties determining phase diagrams. Thus, information about the lipid characteristics may be obtained either from measurements of material properties or determinations of phase diagrams of the lipid system.

It has become quite apparent from the literature that lipids which form reversed hexagonal (H_{II}) and cubic phases are important in modulating the activity of membrane proteins [5,11,21–28]. Indeed it has been found that *A. laidlawii* strain A-EF22 adjusts the composition of its membranes in order to stay relatively close to an L_α to non-lamellar phase transition [9,29]. From these and other experimental data it has been suggested that some properties, such as the tendency of lipid monolayers to form curved surfaces, may play an important role for membrane functioning [5,16,25,29,30].

The mycoplasma *A. laidlawii* strain A-EF22 is a simple bacterium that lacks a cell wall and internal membrane structures [8]. It is an attractive organism for studies of the physical chemistry of membranes, since it readily incorporates exogenous fatty acids as acyl chains of its membrane lipids. Furthermore, since the organism cannot synthesize essential unsaturated fatty acids, and since the synthesis of saturated fatty acids can be nearly completely inhibited, the make-up of the acyl chains is largely under experimental control. One therefore has the advantage to biosynthetically incorporate labelled fatty acids into the membrane in order to carry out spectroscopic investigations such as ^{2}H-NMR [11]. In the cell membrane of strain A-EF22 there are seven classes of lipids [8,31–33]; the two glucolipids 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol (MGlcDAG) and 1,2-diacyl-3-*O*-(α-D-glucopyranosyl-(1 → 2)-*O*-α-D-glucopyranosyl)-*sn*-glycerol (DGlcDAG) constitute the major fraction. A third glucolipid is synthesized by this strain when large amounts of saturated, straight-chain fatty acids (16 to 18 carbon atoms) are incorporated into the lipids [34,35]. The structure of this lipid was determined to be 1,2-diacyl-3-*O*-(6-*O*-acyl-(α-D-glucopyranosyl))-*sn*-glycerol (MAMGlcDAG) [32]. Accordingly, growth of this organism in the presence of certain high-melting fatty acids induces synthesis of a membrane lipid with a large hydrophobic part and a relatively small hydrophilic headgroup.

In a large number of studies we have shown (see [8,13] for recent reviews) that the phase behaviour of the lipids plays a crucial role in the regulation of the membrane lipid composition. The potential ability of the lipids, e.g., MGlcDAG, to form a variety of non-lamellar liquid crystalline phases is of great importance. The most general result can be summarized as follows. An increase in for example the growth temperature, or in the acyl chain unsaturation, can be predicted to induce a decrease in the ratio MGlcDAG/DGlcDAG, since a stable bilayer cannot incorporate large amounts of MGlcDAG. In the same way, introduction of hydrophobic molecules like alkanes in the membrane also leads to a decrease in this ratio [36,37].

In our early model of the lipid regulation in the membrane of *A. laidlawii* strain A-EF22 it was suggested that MGlcDAG is the primary lipid responsible for the mechanism behind the regulation, since this was the only lipid, known at that time, that forms reversed non-lamellar phases [29]. However, this proposal has to be modified, since there are at least two lipids forming other than bilayer structures (MGlcDAG and MAMGlcDAG), and most probably there are even three lipids [33]. Obviously, the lipid regulation is more complicated than originally suggested. However, the basic ideas suggesting that the regulation is governed by the physico-chemical properties of the lipids still appear to be sound. In our previous studies the phase equilibria were predominantly obtained on either single lipids or total membrane lipid extracts. However, in order to get a more quantitative measure of the efficacy of MGlcDAG and MAMGlcDAG to induce non-lamellar phases, we have performed some crucial phase studies. Thus, by using ^{2}H-NMR on α-deuterated fatty acids, the ability of MGlcDAG and MAMGlcDAG to induce non-bilayer structures in a lipid mixture with an in vivo composition, extracted from the membrane of *A. laidlawii* strain A-EF22, has been determined.

2. Materials and methods

Bovine serum albumin (BSA), fraction V, was purchased from Boehringer Mannheim, Mannheim, Germany, and tryptose was obtained from Difco Laboratories, Detroit, MI. The radioactively labelled fatty acids were supplied from Du Pont NEN. The α-deuterated palmitic acid was synthesized according to a procedure described in Ref. [38].

2.1. Growth and harvest of cells

A. laidlawii strain A-EF22 was grown in 40 l of a lipid-depleted BSA/tryptose medium [39]. Lipid-depletion of tryptose and BSA was performed as described by Razin and Rottem [40] with the following modifications: the second extraction step of tryptose, with a mixture of chloroform and methanol, was allowed to run over night, and the third extraction with chloroform-methanol was followed by an extraction with diethyl ether running over night. The medium was supplemented with 120 μM α-deuterated palmitic (16:0) acid and 30 μM oleic (18:1c) acid. In parallel with the 40 l batch, cells were grown in 160 ml of medium also supplemented with 15 μCi/l ^{3}H-16:0 and 5 μCi/l ^{14}C-18:1c, for the determination of the polar headgroup composition of the membrane lipids. The cultures were grown for 22 h at 30°C. The cells were harvested by centrifugation (17000 × g at 5°C for 30 min) and washed once in a β-buffer.

2.2. Lipid purification and analysis

The extraction of the membrane lipids from the cell pellets, and the purification of the lipid extracts on a Sephadex G-25 Fine column, were performed as described in [39]. In order to remove any gel particles present in the eluate from the Sephadex column, one volume of benzene was added to the lipid extract; the solution was centrifuged in a bench centrifuge, and the supernatant was collected. Before isolation of MAMGlcDAG and MGlcDAG from the lipid extract obtained from the 40 l batch, divalent cations were removed with an EGTA extraction method described earlier [9].

The lipid extract was applied to a silicic acid (Bio-Sil HA minus 325 mesh, Bio-Rad Laboratories, Richmond, CA) column in order to isolate MAMGlcDAG and MGlcDAG. Pigments, neutral lipids and free fatty acids were first eluted with chloroform. MAMGlcDAG and MGlcDAG were then eluted with chloroform-methanol (97:3, v/v) and chloroform-methanol (95:5 to 90:10, v/v), respectively. The remaining lipids were finally eluted with methanol.

A small portion of the total lipid extract was used to determine the acyl chain composition of the lipids. 10 mg of the lipids was applied as a stripe to two pre-coated Silica gel 60 thin-layer chromatography (TLC) glass plates with a layer thickness of 0.25 mm (Merck, Darmstadt, Germany). The chromatograms were developed with chloroform-methanol-water (60:30:4.5). The lipids were visualized with Rhodamine 6G and were then extracted from the TLC gel as described previously [41]. The lipid acyl chains were converted to their methyl esters, and the acyl chain composition was determined by gas-liquid chromatography as described earlier [41].

The polar headgroup composition of the membrane lipids was determined from the cells grown with ^{3}H and ^{14}C labelled fatty acids. The purified lipids were first separated on a pre-coated Silica gel 60 TLC aluminium sheet with a layer thickness of 0.2 mm (Merck, Darmstadt, Germany); the chromatogram was developed with chloroform-methanol-water (60:30:4.5). The TLC gel with each lipid was scraped off the sheet into liquid scintillation vials. The gel was treated with 0.5 ml Soluene™ at 37°C for 1 h. Thereafter 50 μl 5 M HCl was added to neutralize the Soluene™. 4.5 ml Picoflour™ 40 was added prior to analysis by liquid scintillation counting with a 1214 Rackbeta Liquid Scintillation Counter LKB Wallac (Wallac, Turku, Finland). The lipids were quantified as described in [42].

2.3. Sample preparation

The lipid samples were dried in 8 mm glass tubes under a stream of N_2 followed by freeze drying at 10^{-3} mmHg until constant weights were obtained. 20% (w/w) of deuterium-depleted water was added and the tubes were flame sealed. The samples were then centrifuged back and forth in the glass tube about ten times at 35°C. Finally, the samples were cycled between −70°C and 25°C in order to mix all components. Since MAMGlcDAG has a very high melting temperature [43], a complete mixing of the lipids in the samples D and E containing MAMGlcDAG was ensured by heating the samples to about 70–80°C until a major isotropic peak in a ^{2}H-NMR spectrum could be detected. After heating the samples, ^{2}H-NMR spectra were recorded as a function of decreasing temperature. This heating procedure was not necessary for the sample F containing 11 mol% MAMGlcDAG, where complete mixing was obtained already at ambient temperature and the NMR spectra were thus recorded with increasing temperature. All the samples containing MGlcDAG were completely mixed at ambient temperature, as judged from the ^{2}H-NMR quadrupole splittings, and these samples were therefore recorded with increasing temperature.

2.4. NMR measurements

^{2}H-NMR spectra were recorded with a Bruker AMX2-500 spectrometer operating in the quadrature detection mode at the frequency 76.77 MHz. A selective high power probe, tuned to deuterium with an 8 mm horizontal solenoid coil (500/8/X, Cryomagnetic Systems, Indianapolis, USA) was utilized. To collect data a phase-cycled quadrupolar echo pulse sequence [44,45] was used with a $\pi/2$-pulse length of 6.4 μs and a 50 μs pulse separation. The recycle time was 150 ms. Typically, 30000 to 40000 transients were collected for each sample and temperature. The temperature was controlled by a Eurotherm B-VT 2000 unit. A temperature calibration curve, based on standard parameter settings, was used to set the desired temperatures. The samples were allowed to equilibrate at each temperature for at least 30 min.

2.5. Analysis of ^{2}H-NMR spectra

The observed quadrupolar splitting, $\Delta\nu_Q$, is directly related to the C-^{2}H bond order parameter, S_{CD}, by the equation [10]

$$\Delta\nu_Q = \frac{3}{2}\left(\frac{e^2qQ}{h}\right)S_{CD}\left(\frac{3\cos^2\theta - 1}{2}\right) \quad (1)$$

where $e^2qQ/h = 170$ kHz and θ is the angle between the bilayer normal and the main magnetic field. In this equation S_{CD} can be defined as

$$S_{CD} \equiv \frac{1}{2}\langle 3\cos^2\beta - 1\rangle \quad (2)$$

in which $\langle\cos^2\beta\rangle$ is the time-average, where β is the angle between the C-^{2}H bond direction and the bilayer normal, the so-called director.

A random distribution of these director axes, normally occurring in liquid crystalline samples where the microcrystallites are randomly oriented, would yield a doublet for each orientation, i.e., a powder pattern [46]. From Eq. (1), it is obvious that for an orientation of the director axis at $\theta = 90°$ with respect to the main magnetic field, the splitting would be one-half of that obtained at $\theta = 0°$. Furthermore, the intensity of the peak corresponding to $\theta = 90°$ would be the greatest and would decline steadily to $\theta = 0°$, where the peak would have the least intensity. This can be visualized by imagining all of the possible orientations as vectors with their arrows on the surface of a sphere. All the vector directions have the same probability. The number of orientations exactly parallel to the magnetic field will be limited to only two, either up or down, whereas the perpendicular orientations can exist anywhere in a plane perpendicular to the magnetic field. Thus, there are more possible orientations perpendicular to the magnetic field. The maximum splitting possible for a ^{2}H-NMR spectrum would be 255 kHz assuming e^2qQ/h equal 170 kHz. This corresponds to the orientation at $\theta = 0°$ of a rigid molecule where $S_{CD} = 1$ (complete ordering). If rapid axial rotation of the hydrocarbon chain occurs, as is often the case for lipids in the gel state, then the maximum splitting is reduced by a factor of two to 127.5 kHz.

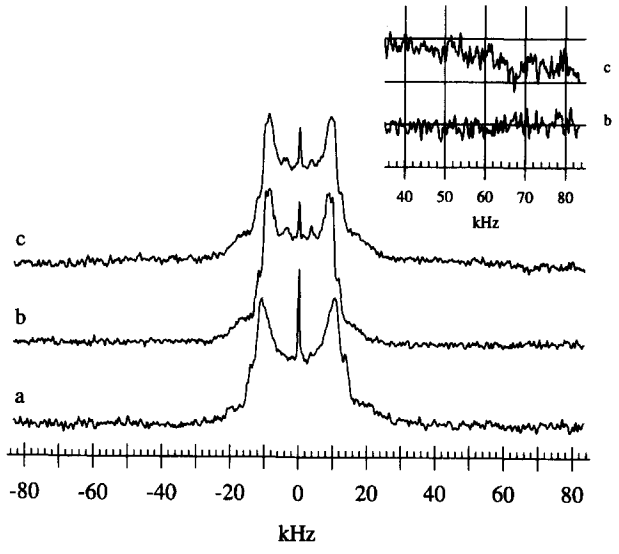

Fig. 1. ^{2}H-NMR spectra of samples E and F in Table 2 at various temperatures. (a) sample F (11 mol% MAMGlcDAG) at 9°C; (b) sample F at 63°C; (c) sample E (30 mol% MAMGlcDAG) at 54°C. Notice the additional quadrupole splitting of about 7 kHz appearing in the spectra b and c originating from an H_{II} phase. The narrow peaks occurring at 0 kHz arise from isotropic water. The inset (spectra magnified 5-times) shows the presence of a gel/crystalline phase in spectrum c.

In the L_α phase the ^{2}H-NMR spectra (Fig. 1a) exhibit the shape of a powder pattern of a sample with uniaxial symmetry. The uniaxiality is caused by the rapid rotational motion about the bilayer normal and the rapid translational diffusion of the lipids [47]. For an H_{II} phase there is a further motional averaging of the quadrupolar splitting due to the translational diffusion of the lipids around the cylindrical axis of the long rod-like aggregates building up the phase structure. If only the difference in geometry between the two phases is considered, this results in a reduction of the splitting by a factor of two [48]. Thus, ^{2}H-NMR of an H_{II} phase gives rise to a splitting with a value that at the most is half of that observed for an L_α phase [12]. However, often the splitting for an H_{II} phase is smaller than that obtained from a geometric assessment, since further motions occur in the H_{II} phase [10]. In the gel phase the translational diffusion and the flexibility of

Table 1
The composition (in mol%) of the lipid extract from the membrane of *Acholeplasma laidlawii* used to prepare all the samples in Table 2

	MAMG1cDAG [a]	MG1cDAG	DG1cDAG	PG	MABGP-DG1cDAG	GPDG1cDAG	Bilayer forming lipids
Mol% of total lipids	5	25	35	24	3	8	70
SFA mol%	78	63	30	38	41	33	34 [b]
UFA mol%	22	37	70	62	59	67	66 [b]
SFA/UFA	3.55	1.70	0.43	0.61	0.69	0.49	0.51 [c]

[a] MAMG1cDAG, monoacylmonoglucosyldiacylglycerol; MG1cDAG, monoglucosyldiacylglycerol; DG1cDAG, diglucosyldiacylglycerol; PG, phosphatidylglycerol; MABGPDG1cDAG, monoacylbisglycerophosphoryldiglucosyldiacylglycerol; GPDG1cDAG, glycerophosphoryldiglucosyldiacylglycerol; SFA, saturated fatty acids; UFA, unsaturated fatty acids.
[b] Weighted values, assuming that DG1cDAG, PG, MABGPDG1cDAG, and GPDG1cDAG are the lipids forming bilayers.
[c] The weighted value of SFA/UFA for all lipids is 0.96.

the acyl chains are strongly reduced and only the rotational motion of the chains remains, causing the quadrupole splitting to be close to 127.5 kHz. Thus, at a transition to a gel phase a large broadening of the splitting is observed in the ^{2}H-NMR spectrum (Fig. 1c) [49–51].

3. Results

3.1. Lipid composition

The polar headgroup and acyl chain composition of the lipid extract with an in vivo composition is shown in Table 1. The nonbilayer-forming lipids MGlcDAG and MAMGlcDAG constituted about 30 mol% of the total lipids. Since the composition of the acyl chains also has a crucial effect on the phase behaviour of the lipids, the contents of saturated (SFA) and unsaturated (UFA) fatty acids are also presented in Table 1. It is seen that the bilayer-forming lipids were rich in UFA, while MGlcDAG and MAMGlcDAG were enriched in SFA. The two fatty acids supplemented to the growth medium (18:1c and α-deuterated 16:0) constituted between 75 and 93 mol% of the total acyl chains in the different lipids.

3.2. ^{2}H-NMR quadrupole splittings

From the total extract lacking MAMGlcDAG and MGlcDAG, and the isolated preparations of these two lipids, a series of samples was prepared in which the fraction of MAMGlcDAG and MGlcDAG was varied from 0 to 45 mol%, and from 0 to 63 mol%, respectively (Table 2). The different contents of the nonbilayer-forming lipids were arbitrarily chosen to 20, 41 and 63 mol% of MGlcDAG, and to 11, 30, and 45 mol% of MAMGlcDAG; these contents were chosen to get an expected large and observable effect. It should be noted that the cell membrane contains at the most about 20 mol% of MAMGlcDAG, while the content of MGlcDAG can vary between approx. 10 and 50 mol% [11,34]. Pigments, free fatty acids and neutral lipids were excluded from all lipid mixtures studied. Finally, all samples contained 20% (w/w) of deuterium-depleted water. The phase equilibria

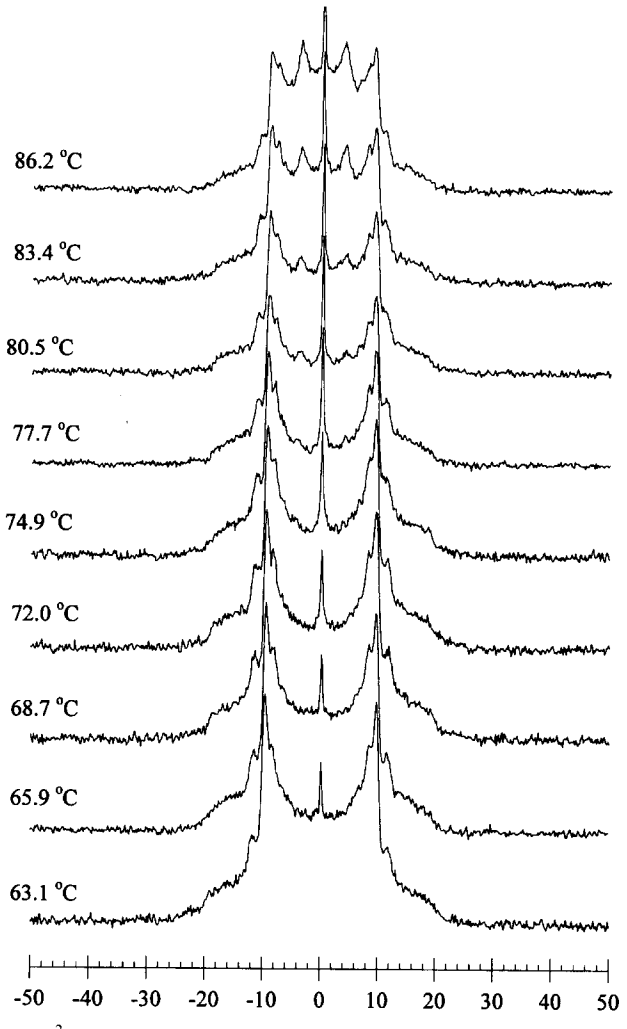

Fig. 2. ^{2}H-NMR spectra of sample C (20 mol% MGlcDAG) in Table 2 at temperatures between 63.1 and 86.2°C. An H_{II} phase giving rise to a quadrupole splitting of about 8 kHz is formed above 75°C.

of the samples were studied as a function of the temperature. Fig. 2 shows a stacked plot of ^{2}H-NMR spectra of the α-deuterated methylene group of the palmitoyl acyl chains of the lipids in the mixture containing 20 mol% MGlcDAG. The spectra have the line-shape of uniaxial powder patterns showing one intense splitting of about 19 kHz flanked by two more splittings of 16 and 22 kHz with very much

Table 2
The lipid composition (in mol%) of the samples studied

Sample	MAMGlcDAG [a]	MGlcDAG	DGlcDAG	PG	MABGP-DGlcDAG	GPDGlcDAG
Reference	–	–	57	29	5	10
A	–	63	18	12	2	4
B	–	41	29	20	3	7
C	–	20	39	27	4	9
D	45	–	36	12	2	4
E	30	–	40	20	3	7
F	11	–	46	28	4	10

[a] See footnote a to Table 1.

lower intensities. The order parameters calculated from Eq. (1) are 0.14 for the intense splitting and 0.12 and 0.17 for the other two. The referred splittings and the order parameters are taken at 78°C, and the temperature dependence of the splittings is found to be rather small; the magnitude of the splittings decreased by about 7% between 63–86°C. The quadrupole splitting of the H_{II} phase at this temperature is 8 kHz, giving an order parameter equal to 0.06. Similarly, a sample at 63°C containing 11 mol% MAMGlcDAG shows an intense splitting of 20 kHz and two splittings of 23 and 17 kHz with lower intensity. These splittings correspond to order parameters equal to 0.15, 0.18 and 0.14, respectively. In general, an α-methylene group at the *sn*-1 position of the glycerol moiety gives rise only to one quadrupole splitting, while it may give rise to two splittings when it is attached to the *sn*-2 position [12,52]. This is due to the fact that the *sn*-2 carbon is chiral, implying that the two deuterons of the α-methylene group are not equivalent and therefore give rise to one splitting each. An unambiguous assignment of all three splittings from lipid mixtures with MGlcDAG or MAMGlcDAG (Figs. 1 and 2) is quite difficult to make, since the samples contain a large number of different lipids. An exact assignment of all the peaks in the NMR spectra is, however, not necessary in this work, since it is focussed on the determinations of phase transitions that can be readily observed without such an information (Fig. 2). Most probably the intense splittings originate from palmitoyl chains on the *sn*-1 position. These splittings have values that are typical for an L_α phase as has been observed in previous work [11,53]. The splittings with the lower intensities could be due to a small fraction of the palmitoyl chains attached to the *sn*-2 position. Other possibilities could be the presence of a large number of different lipids or the fact that three of the lipids have a third acyl chain [31–33].

3.3. Phase equilibria

The sample lacking MAMGlcDAG as well as MGlcDAG was used as a reference sample (Table 2). This sample did not show any phase transition up to 90°C, i.e., the L_α phase was stable up to at least 90°C. Higher temperatures were not investigated, since the lipids can be expected to decompose. Figs. 1 and 2 show typical ^{2}H-NMR spectra of quadrupole splittings for phases at different temperatures and fractions of MAMGlcDAG or MGlcDAG. A sample with 11 mol% MAMGlcDAG forms an L_α phase even at low temperatures such as 9°C (Fig. 1a) and this phase was present up to about 53°C. Fig. 1b and c show that at higher temperatures an H_{II} phase is formed in addition to the L_α phase for the samples E and F (Table 2). With large fractions of MAMGlcDAG (samples D and E) a gel/crystalline phase is observed even at high temperatures having a quadrupole splitting equal to about 127 kHz (Fig. 1c), which is close to the theoretical value for a fast rotating all *trans* acyl chain in a bilayer (see Section 2). Above approx. 52°C three or four phases were in equilibrium, namely gel, L_α, H_{II}, and isotropic phases.

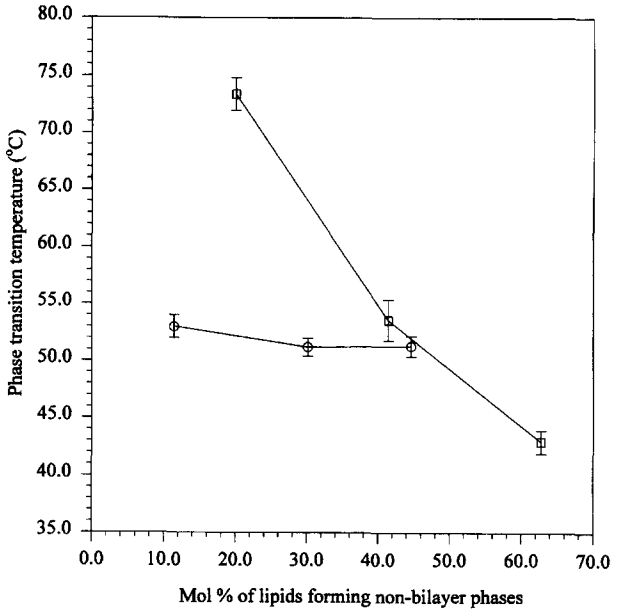

Fig. 3. The temperature for the L_α to non-lamellar phase transition as a function of the content of MGlcDAG (□) and MAMGlcDAG (○) in the lipids extracted from the membrane of *Acholeplasma laidlawii*.

Fig. 3 summarises the phase transition temperatures as a function of the fractions of MAMGlcDAG and MGlcDAG in the *A. laidlawii* lipid samples. The effect of MGlcDAG on the transition temperature is very strong, while this temperature is nearly independent of the concentration of MAMGlcDAG in the lipid mixtures. However, the impact of MAMGlcDAG at the lowest, and physiologically relevant, content highly exceeds that of MGlcDAG.

4. Discussion

A physico-chemical hypothesis was presented about 15 years ago, attempting to give an explanation of the regulation of the membrane lipid composition in *A. laidlawii* strain A-EF22 [54]. In the formulation of our hypothesis at that time the shape of the lipid molecules was emphasised. The theoretical basis was the model for the self-assembly of amphiphiles developed by Tanford [55] and by Israelachvili and colleagues [56,57]. The formation and the shape of lipid aggregates are determined by the counterbalance between attractive and repulsive interactions. Thus, lipid membranes comprise an interplay between many different forces of steric, van der Waals and electrostatic origin. It is proposed that the packing of the lipids into different aggregates is determined by the hydrophobic volume, the interfacial area and the hydrocarbon chain

length of the lipid molecules. Accordingly, bilayers are built up of cylindrical-like molecules and divergence from this molecular shape results in the formation of non-lamellar phases, like cubic and H_{II} phases [5,20,54,57].

Though the central notion is still proper, our model of the lipid regulation has been rephrased and improved since then (for obvious reasons it is not possible to actually *measure* the molecular shape of a separate lipid in an aggregate) [1,5,13,43]. Thus, we state that the cells strive to maintain a balance between the membrane lipids constituting a bilayer structure and those forming reversed non-lamellar aggregates. In this simple model, there is a requirement of a foreordained packing or curvature of the lipids in the membrane, which strongly depends on the phase behaviour of the lipids. The main idea behind the hypothesis is that for the membrane to function these properties of packing and curvature have to be kept within a narrow interval. If there is a perturbation of the molecular packing or elastic stress in the membrane, there will be a compensating change in the lipid composition, striving to maintain the balance between the cylindrical-like and the wedge-shaped lipid molecules building up most membranes.

A more general theory of the physical properties of lipid bilayers, in the sense that it can be applied to more complex systems where phase behaviour is difficult to obtain, is based on a phenomenological model of material physics introduced by Helfrich [58]. The stability of the lipid aggregate in his approach is dominated by an elastic free energy of curvature, and a concept with relations to the molecular packing is utilized, namely the so-called spontaneous curvature of the lipid monolayer. One of the important material properties is the curvature elastic stress, and this stress within the bilayer can according to Helfrich be formulated as [58,59]

$$g_c = \kappa(H - H_0)^2 + \bar{\kappa}K \qquad (3)$$

in which g_c represents the free energy of curvature per unit area. Here $H = (c_1 + c_2)/2$ is the mean curvature; H_0 the spontaneous curvature (a material property); c_1 and c_2 the two principal curvatures; K the Gaussian curvature; and κ and $\bar{\kappa}$ are the bending rigidity and Gaussian curvature modulus, respectively. Membrane stability may be understood as a balance between the energy required to bend the bilayer and the energy gained by avoiding exposure of the hydrophobic interior of the bilayer to water. Bending a surface away from its spontaneous or equilibrium curvature then would be an energetically unfavorable state, and the above expression quantifies the energetic cost of such a bending. If H_0 is non-zero, which is proposed for lipids with small headgroups, such as MAMGlcDAG and MGlcDAG, then the most favorable state of an individual monolayer would be curved. Therefore, packing such molecules into a planar bilayer structure would be energetically unfavorable with respect to the curvature. It is the presence of this curvature energy in L_α phases that has generated interest in the properties of lipid H_{II} and cubic phases [15,16,30,37,60].

Several techniques have been used to probe the structure of lipid/water systems, among them different NMR techniques. ^{2}H- and ^{31}P-NMR spectroscopy have been used to conveniently map out the phase diagram of different lipid systems [61–64]. Moreover, ^{2}H-NMR spectroscopy has emerged as a powerful tool in characterizing headgroup and acyl chain orientations of lipids and membranes [9–11,39,43,51,65]. Its advantage lies in the fact that, unlike many other techniques, ^{2}H-NMR provides information at the molecular level about both orientational order and dynamics. From the experimental data estimates of physical properties in the L_α phase can be made in terms of simple statistical models [10,66–68]. The cross-sectional chain area is related to the mean area occupied by a lipid molecule at the membrane lipid-water interface. This area in turn is important in determination of the curvature free energy of the lipid aggregate [10,11,16,69], which reflects various contributions to the energetics of lipid systems and may play a role in regulating membrane protein function [5,24–26,70]. Analysis of ^{2}H-NMR data has indicated that significant differences in packing and dynamics exist between the H_{II} and the L_α phases [12]. These differences can be related to the radius of curvature in the H_{II} phase and can help to understand the role different phases play in biological systems.

In a recent investigation [43] the phase equilibria of the individual lipids MGlcDAG and MAMGlcDAG, isolated from *A. laidlawii* strain A-EF22, were compared. It was found that hydrated MAMGlcDAG does not form any liquid crystalline phases, but a gel/crystalline phase is stable at physiological temperatures and a reversed micellar (L_2) phase is formed at about 80°C. In this study, and in an earlier investigation [39], it was also found that MGlcDAG enriched in SFA prefers to form an L_α phase at physiological temperatures. This can be understood from the change in the shape of the lipid, since the difference in cross sectional areas between the polar headgroup and the apolar hydrocarbon part will decrease. Thus, the shape of MGlcDAG will change towards a cylinder. Therefore, MGlcDAG will reduce its capability to induce reversed non-lamellar phases, and bacteria grown with saturated fatty acids will consequently lack this device for keeping the balance between lipids forming lamellar and non-lamellar structures. However, the cell is able to resolve this problem by synthesising MAMGlcDAG, which has three acyl chains, and is therefore anticipated to be even more potent to form non-bilayer structures than MGlcDAG. Thus, it is hypothesised that MAMGlcDAG assists MGlcDAG in maintaining an optimal packing or elastic bending force (negative curvature) of the lipids in the membrane [43]. The main purpose of the present work is to substantiate this proposal by studying the effect of the two non-lamellar forming lipids on the structure of total lipid extracts from the membrane of *A. laidlawii* strain A-EF22.

A mixture of the lipids DGlcDAG, phosphatidylglycerol (PG), glycerophosphoryldiglucosyldiacylglycerol (GPDGlcDAG) and monoacylbisglycerophosphoryldiglucosyldiacylglycerol (MABGPDGlcDAG) (Table 1), that individually only form an L_α phase, is found to remain in the L_α state up to at least 90°C. This pertains although these lipids have a much higher content of UFA than MGlcDAG and MAMGlcDAG (Table 1). Addition of increasing amounts of MGlcDAG to this lipid mixture results in a transition from an L_α to an H_{II} phase (Figs. 2 and 3). The phase transition temperature is as high as about 75°C for an incorporation of 20 mol% of MGlcDAG and at 63 mol% MGlcDAG the phase transition temperature has gone down to about 43°C. Thus, the transition temperature decreases steeply as a function of the MGlcDAG content (Fig. 3). This observation lends further support to our proposal [29] that MGlcDAG is a potent regulator of the balance between lamellar and non-lamellar phases.

Addition of MAMGlcDAG to the reference mixture forming an L_α phase resulted in a transition to non-lamellar (H_{II} and isotropic) phases at about 51–53°C (Fig. 1b, c and 3). However, there are two important differences compared with the result obtained for MGlcDAG; there is no steep dependence of the phase transition temperature on the concentration of MAMGlcDAG (above 11 mol%); and the phase transition occurs at a much lower temperature with a small fraction of MAMGlcDAG. These findings can be explained as follows: the lack of a concentration dependence within the interval studied is most probably due to the fact that the solubility of MAMGlcDAG in an L_α phase is limited. This conclusion is supported by the large ^{2}H-NMR quadrupole splittings obtained from the samples in which the concentrations of MAMGlcDAG are 30 and 45 mol%. The large splittings correspond to a gel/crystalline phase, which is in equilibrium with an L_α phase at high MAMGlcDAG contents (Fig. 1c). It is interesting to note that the highest content of MAMGlcDAG in the membrane of *A. laidlawii* strain A-EF22 is about 20 mol% [34]. Thus, it can be concluded that concentrations of MAMGlcDAG above approx. 20 mol% is probably excluded from the fluid bilayer as a separate gel/crystalline phase. Therefore, an increase in the MAMGlcDAG content above 20 mol% in the bilayer will not further decrease the phase transition temperature (Fig. 3). Furthermore, no gel/crystalline phase is observed even at 9°C for the sample with 11 mol% MAMGlcDAG (Fig. 1a), although pure MAMGlcDAG has a transition temperature from gel/crystalline to a fluid isotropic phase at about 80°C [43].

In conclusion we have shown that the two lipids MGlcDAG and MAMGlcDAG are capable to induce non-lamellar phases in the lipid membrane of *A. laidlawii* strain A-EF22. It appears to be necessary for this organism to always have at least one lipid forming non-bilayer structures present in the membrane. The result of this work can be taken as further strong support for our model describing the physico-chemical properties of the bilayer and the lipid regulation in a bacterial membrane. Thus, the phase behaviour, closely related to the lipid packing and the elastic bending properties of the bilayer, plays a crucial role for the understanding of the functioning cell membrane. By knowing the phase behaviour of the lipids in the membrane we are, in fact, in a position to predict how the physico-chemical properties of the bilayer will participate and affect a biological membrane process. This will be of inestimable value for the understanding of for example the mechanisms behind, and the functions of, many membrane-bound enzymes such as lipid synthases, although the lipid regulation in *A. laidlawii* is more complicated than originally suggested. Thus, the basic ideas proposing that the regulation is somehow governed by the phase properties of the lipids still appear to be sound, although the number and function of the enzymes responsible for the regulation are more obscure than previously thought.

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