Acholeplasma laidlawii B Membranes Contain a Lipid (Glycerylphosphoryldiglucosyldiacylglycerol) Which Forms Micelles Rather Than Lamellar or Reversed Phases When Dispersed in Water†

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ABSTRACT: It has been proposed that each of the lipids from the *Acholeplasma laidlawii* membrane prefers to form either a lamellar or a reversed cubic or hexagonal phase when dispersed in excess water at physiologically relevant temperatures and ionic strengths. In this study, we have reinvestigated the thermotropic phase behavior of all the major membrane lipids from *A. laidlawii* B membranes derived from cells grown in equimolar palmitic and elaidic acids. We confirm that phosphatidylglycerol (PG) and diglucosyldiacylglycerol (DGDG) from such membranes do indeed form only lamellar phases over the temperature range 5−80 °C. We also confirm that the monoglucosyldiacylglycerol (MGDG) and acyl polyprenyl glucoside (APG) exist in lamellar phases at lower temperatures but do form reversed phases at higher temperatures. However, we present here optical, differential scanning calorimetric, quasielastic light scattering, and ^{2}H- and ^{31}P-nuclear magnetic resonance spectroscopic evidence indicating that one lipid component of the *A. laidlawii* B membrane, namely, glycerylphosphoryldiglucosyldiacylglycerol (GPDGDG), actually forms normal micellar rather than lamellar or reversed phases when dispersed in excess water at physiological temperatures. To the best of our knowledge, this is the first demonstration of the existence of a micellar phase-preferring lipid in a prokaryotic cell membrane, and only the second demonstration of the existence of a micellar phase-forming lipid in any biological membrane. We also show that GPDGDG levels change greatly depending on the fatty acid composition of the membrane lipids of this organism. Specifically, we demonstrate that GPDGDG levels can vary from as low as 3 mol % in *A. laidlawii* cells grown in saturated fatty acids to nearly 30 mol % in cells enriched in polyunsaturated or short chain methyl isobranched fatty acids. Moreover, in the accompanying paper [Foht et al. (1995) *Biochemistry 34,* 13811−13817], we demonstrate that the existence of this micellar phase-forming lipid is likely to have a profound effect on lipid organization and phase preference in this organism, even when present in relatively small quantities. Finally, we show that the MGDG/DGDG ratio, previously used to estimate overall membrane lipid phase preference of *A. laidlawii* A and B, is inadequate to this task, in part because the existence of varying quantities of GPDGDG is not accounted for in this ratio.

The lipid components of all biological membranes thus far studied appear to be arranged exclusively in the lamellar or bilayer phase under physiologically relevant conditions of temperature, ionic strength, and hydration (Jain, 1988; Gennis, 1989). Nevertheless, many biological membranes contain one or more lipid components which, in isolation, prefer to form reversed hexagonal (H_{II})[1] or reversed cubic (Q_{II}) phases (Cullis et al., 1983; Rilfors et al., 1984; Gruner et al., 1985; Gruner, 1992). Although these reversed phase-preferring lipids may be major components of some biological membranes, the structural and functional roles of such lipids have not been clearly established. Some workers have presented evidence that the transient formation of reversed nonlamellar lipid phases induced by these lipids may play a key role in processes such as membrane fusion (Verkleij et al., 1979; Siegel, 1986a,b), in the transmembrane movement of ions and macromolecules, or in the function of membrane-associated enzymes (Cullis et al., 1983; Rilfors et al., 1984; Gruner et al., 1985; Lindblom & Rilfors, 1989; Gruner, 1992). Other workers have presented evidence that the role of reversed phase-forming lipids is to impart special (though as yet incompletely defined) properties to the lipid bilayer phase rather than to induce the actual formation of nonlamellar lipid structures (Gruner et al., 1985; Gruner, 1992; Hui, 1987). Defining the specific structural and functional roles of reversed phase-preferring lipids in particular, and of lipid compositional diversity in general, remains a major unresolved problem in membrane biology.

The phase preference of different membrane lipids can be related to the overall shape of the lipid molecules under various environmental conditions. The molecular shape is in turn dependent on the relative effective sizes of the polar and nonpolar regions of the amphiphilic lipid molecules, in particular, on the relative cross-sectional areas occupied by the polar headgroup and nonpolar hydrocarbon chains. The effective cross-sectional area of the polar headgroup depends

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[1] Abbreviations: H_{II}, reversed hexagonal phase; Q_{II}, reversed cubic phase; L_c, lamellar crystalline phase; L_β, lamellar gel phase; L_α, lamellar liquid-crystalline phase; MGDG, monoglucosyldiacylglycerol; DGDG, diglucosyldiacylglycerol; PG, phosphatidylglycerol; APG, acyl polyprenyl glucoside; GPDGDG, glycerylphosphoryldiglucosyldiacylglycerol; DSC, differential scanning calorimetry; QELS, quasielastic light scattering; NMR, nuclear magnetic resonance spectroscopy.

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on its volume, charge, degree of hydration, and orientational and motional freedom and is only modestly affected by changes in temperature. The effective cross-sectional area of the apolar region of the lipid molecule depends on the structure (i.e., degree of unsaturation) and the length of the hydrocarbon chains. In the case of the nonpolar region of the lipid molecule, temperature is an important variable, since the effective cross-sectional area occupied by the hydrocarbon chains increases more markedly with temperature, particularly when a change in temperature induces a gel-to-liquid-crystalline phase transition.

If the effective cross-sectional area occupied by the polar headgroup significantly exceeds that occupied by the nonpolar region of the lipid molecule, such as is the case for some detergents and for lysophospholipids above their phase transition temperature, then these wedge-shaped molecules aggregate in water to form spherical or elliptical micelles. If the relative areas occupied by the polar headgroup and the hydrocarbon chains are roughly equal, then these cylindrically shaped molecules form lamellar phases. This is the phase formed, for example, by phosphatidylcholine and most other diacylglycerolipids. Finally, if the relative cross-sectional area of the polar headgroup is less than that of the hydrocarbon chains, as is the case for some phosphatidylethanolamines, for example, then these "inverted" wedge-shaped molecules may aggregate in water to form a reversed cubic or hexagonal phase. Some workers have suggested that cholesterol molecules may also have an inverted wedge shape, since the cross-sectional area occupied by the small polar "headgroup" is much less than that occupied by the steroid ring system (but see Lewis et al., 1995). The relationship between molecular shape and lipid phase structure has been discussed at length in a number of reviews (Israelachvili et al., 1980; Cullis et al., 1983; Rilfors et al., 1984; Gruner et al., 1985; Gruner, 1992; Lewis et al., 1995).

Acholeplasma laidlawii is a simple, cell wall-less procaryotic microorganism that possesses a number of features which make it an attractive system for studying the structural and functional roles of lipids in biological membranes (McElhaney, 1984, 1989). Two particularly useful features of this organism for such studies are the ability to dramatically alter its membrane lipid fatty acid composition and cholesterol content and its growth temperature. Utilizing these features, it has been shown that alterations in membrane lipid fatty acid composition, cholesterol content, and growth temperature induce marked changes in the quantitative distribution of the major glycolipids of the *A. laidlawii* A membrane (Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1978; Rilfors, 1980; Wieslander et al., 1981). In particular, the MGDG to DGDG ratio was shown to increase with an increase in the degree of unsaturation of the membrane lipid hydrocarbon chains, with an increase in cholesterol incorporation, and with an increase in temperature. Since aqueous dispersions of MGDG prefer to form the H_{II} or Q_{II} phase at higher temperatures, particularly if its fatty acyl chains are unsaturated or cholesterol is present, while the DGDG exists exclusively in the lamellar state under these conditions (Wieslander et al., 1978, 1981a,b; Khan et al., 1981; Lindblom et al., 1986), these characteristic shifts in the MGDG/DGDG ratio were interpreted as a compensatory mechanism to maintain an optimal balance of lamellar- and reverse hexagonal phase-forming lipids. Specifically, these workers postulate that this organism maintains its membrane lipid in a lamellar liquid-crystalline state at any particular growth temperature, but in close proximity to a lamellar/reversed cubic or hexagonal phase transition, by appropriate biochemical alterations in the polar headgroup composition of its membrane lipids. Although this hypothesis seems to be relatively well supported in *A. laidlawii* A [Christiansson et al., 1981, 1985; Clementz & Christiansson, 1986; Wieslander et al., 1986; Clementz et al., 1987; Rilfors et al., 1987; Wieslander & Selstam, 1987; but see discussion in McElhaney (1984, 1989) and Wieslander et al. (1994)], the results of similar studies in the closely related *A. laidlawii* B are not fully compatible with this hypothesis (Silvius et al., 1980; Bhakoo & McElhaney, 1988).

In the studies discussed above, evidence was presented that aqueous dispersions of the major phospholipid of *A. laidlawii* A membranes, PG, like DGDG, exists exclusively in the lamellar state at physiological temperatures, irrespective of fatty acid composition or cholesterol content, and that this is true also for all of the "minor" lipids present in the membrane of this organism (Wieslander et al., 1978, 1981a,b; Khan et al., 1981; Lindblom et al., 1986). However, we have recently shown that a normally minor lipid component of the *A. laidlawii* B membrane, namely, APG, can become the predominant lipid component in cells from membranes enriched in higher melting fatty acids (Bhakoo et al., 1987) and that this lipid, like MGDG, prefers to form an H_{II} phase at higher temperatures (Lewis et al., 1990). Moreover, additional reversed phase-preferring acylated glycolipids have recently been characterized in *A. laidlawii* A (Lindblom et al., 1993). In this paper we show that another "minor" lipid component, namely, GPDGDG, can also become a major component when cells are enriched in certain exogenous fatty acids and that GPDGDG in isolation prefers to form a normal *micellar phase* under physiological conditions. To our knowledge, this is the first report of the presence of a micelle-forming lipid in a biological membrane from a prokaryotic microorganism, and only the second report of the existence of a micellar-forming lipid in any biological membrane.

MATERIALS AND METHODS

A. laidlawii B cells were grown at 37 °C in a lipid-poor growth medium and harvested at mid-log phase as described previously (Silvius & McElhaney, 1978; Silvius et al., 1980). In some cases, avidin, an inhibitor of *de novo* fatty acid biosynthesis and exogenous fatty acid chain elongation in this organism, was added to the growth medium, as was an exogenous fatty acid or pair of fatty acids. The polar lipids were extracted from isolated membranes and the individual lipid components purified, separated, and quantitated as already reported (Monck et al., 1992). DSC thermograms were recorded with a Perkin Elmer DSC-2C low-sensitivity scanning instrument (Perkin-Elmer Corp., Norwalk, CT) operating at scan rates of 1 °C/min or with a Hart Scientific high-sensitivity instrument (Pleasant Groove, UT) operating at scan rates near 30 °C/h, as described earlier (Lewis et al., 1990). The ^{2}H-NMR spectra were obtained on a home-built spectrometer operating at 46.175 MHz using the quadrupolar echo pulse sequence as described in detail earlier (Monck et al., 1992). ^{31}P-NMR spectra were recorded with a Varian Unity 300 spectrometer (Varian Instruments, Palo Alto, CA) operating at 121.42 MHz for ^{31}P using the data acquisition and data processing parameters described by Lewis et al.

Table 1: Lipid Polar Headgroup Compositions of Membranes from *A. laidlawii* B Cells Biosynthetically Enriched in Various Endogenous and Exogenous Fatty Acids[a]

media supplementations		lipid polar headgroup composition (mol %)				
fatty acid	avidin	MGDG	DGDG	PG	GPDGDG	APG
none	−	34.2 ± 2.3	31.3 ± 1.8	28.2 ± 1.9	3.0 ± 0.5	2.1 ± 0.6
16:0[b]	−	7.6 ± 2.0	7.2 ± 1.5	16.2 ± 4.2	8.6 ± 2.7	60.5 ± 5.5
16:0 + 18:1_t	+	36.1 ± 2.5	33.2 ± 1.2	23.1 ± 3.4	7.7 ± 2.3	
16:0 + 18:1_c	+	33.2 ± 2.6	28.6 ± 0.2	21.2 ± 1.0	16.7 ± 0.9	
16:0 + 18:2_{cc}	+	29.6 ± 2.2	23.6 ± 2.8	24.0 ± 1.3	22.3 ± 1.6	
14:0_i	+	46.8 ± 4.7	2.8 ± 1.3	21.4 ± 3.8	29.1 ± 0.4	
15:0_i	+	47.1 ± 2.9	8.5 ± 3.1	17.2 ± 3.1	27.2 ± 2.6	
16:0_i	+	50.8 ± 2.0	9.0 ± 2.4	25.3 ± 0.1	14.9 ± 0.3	
17:0_i	+	42.1 ± 9.6	11.9 ± 1.7	14.7 ± 0.8	2.7 ± 0.3	28.6 ± 10.0

[a] Values presented are the arithmatic means (averages), and average deviation from the means, of three independent determinations of the polar headgroup compositions for each fatty acid composition studied. [b] Fatty acids are designated by the number of carbon atoms followed by the number of double bonds, if any, present in the hydrocarbon chains; the subscripts c and t denote the *cis* and *trans* configurations, respectively, of these double bonds. The subscript i indicates an additional methyl group attached to the penultimate carbon atom (an isobranched fatty acid).

(1988). The QELS experiments were performed on a Brookhaven Model BI-90 light-scattering instrument equipped with a Model BI-2030AT digital correlator (Brookhaven Instruments Corp., Brookhaven, NY). The QELS measurements were performed with argon ion laser light of wavelength 488 nm at an angle of 78.0°. The size distributions of the obviously bimodal population of particles were obtained using non-negatively constrained least square and double exponential analysis. For the DSC and NMR spectroscopic measurements performed in the absence of calcium ions, lipid samples were dispersed in a buffer containing 50 mM Tris, 100 mM NaCl, and 10 mM EDTA (pH 7.4). For those DSC and NMR measurements performed in the presence of Ca^{2+}, 10 mM $CaCl_2$ replaced the EDTA. For the QELS experiment the GPDGDG was dissolved in a buffer containing 5 mM NaH_2PO_4 and 50 mM NaCl (pH 7.4). Typically, lipid concentrations were about 1 mg/mL in the QELS experiments, 3−5 mg/mL in the ^{31}P-NMR and high-sensitivity DSC experiments, 10 mg/mL in the ^{2}H-NMR experiments, and 40−60 mg/mL in the low-sensitivity DSC studies.

RESULTS

The Influence of Fatty Acid Compositional Variations on GPDGDG Levels. Typical lipid polar headgroup compositions of *A. laidlawii* B membranes biosynthetically enriched with a variety of exogenous fatty acids are presented in Table 1. In membranes from cells grown without avidin or an exogenous fatty acid, and which contain primarily (~95 mol %) endogenous palmitic and myristic acids, the major lipid components are MGDG, DGDG, and PG, with only a small amount (3.0 mol %) of GPDGDG being present. Similarly, in membranes from cells grown without avidin but enriched (~75 mol %) in exogenous palmitic acid, GPDGDG is also present in small amounts, but in this case APG has replaced the neutral glycolipids and PG as the major lipid species. When cells are grown in the presence of avidin and equimolar amounts of palmitic and an unsaturated fatty acid, the GPDGDG levels rise significantly. Specifically, GPDGDG levels are less than 8 mol % when the unsaturated fatty acid is elaidate, are about 17 mol % when oleate is present, and are over 22 mol % when the unsaturated fatty acid is linolenate. In membranes made nearly homogeneous in a homologous series of methyl isobranched fatty acids, GPDGDG levels are quite high in the shorter chain members of this series but progressively decline as chain length increases. Interestingly, a similar trend is not noted in membranes enriched in members of the methyl anteisobranched and ω-cyclohexyl fatty acid series, where GPDGDG levels remain low (<5 mol %) and essentially independent of chain length (data not presented). These results indicate that the biosynthetic incorporation of exogenous short chain methyl isobranched or long chain *cis*-unsaturated fatty acids markedly elevates the relative amount of GPDGDG synthesized by this organism. Since GPDGDG levels can become significant under these circumstances, the physical properties of this lipid, as well as the other major lipid components extracted from membranes derived from cells grown in equimolar amounts of perdeuterated palmitic and nonperdeuterated elaidic acid, were studied by several physical techniques. Although GPDGDG levels are relatively low in such cells, this fatty acid composition was chosen to produce membrane lipid molecular species with phase transition temperatures in a convenient range for measurement by DSC and to permit ^{2}H-NMR as well as ^{31}P-NMR spectroscopic characterizations of GPDGDG phase behavior.

Physical Studies of Aqueous Dispersions of GPDGDG. In preparing aqueous dispersions of the individual lipid components of the *A. laidlawii* B membrane, the macroscopic behavior of the GPDGDG component was observed to differ from that of the other polar lipids. Specifically, whereas aqueous dispersions of APG, MGDG, DGDG, and PG are opaque at temperatures of 0−55 °C, aqueous dispersions of GPDGDG form completely clear, viscous solutions at these temperatures, even with concentrations of lipid as high as 50 mg/mL. Since APG, MGDG, DGDG, and PG form lamellar phases over this temperature range, this observation suggests that the GPDGDG does not. Although translucent aqueous dispersions can be formed by APG and MGDG at higher temperatures (where they exist in the H_{II} phase), the large size of the GPDGDG polar headgroup would seem to preclude the formation of a reversed phase, particularly at lower temperatures. Moreover, at these higher temperatures, GPDGDG dispersions appear clear rather than translucent. The macroscopic behavior of the GPDGDG is compatible, however, with the formation of lipid micelles.

The anomalous behavior of GPDGDG is also manifest in the thermotropic phase behavior of this lipid. As shown in Figure 1A−D, aqueous dispersions of all of the major membrane lipids of *A. laidlawii* except GPDGDG exhibit at least one endothermic phase transition in the temperature

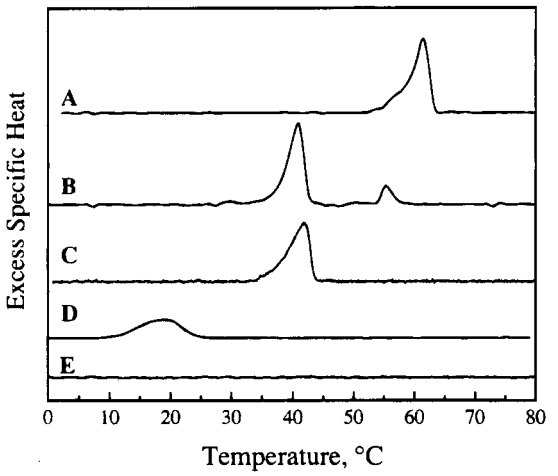

FIGURE 1: Low-sensitivity DSC heating thermograms of aqueous dispersions of each of the major membrane lipids from *A. laidlawii* B cells grown in media supplemented with an equimolar mixture of elaidic acid and perdeuterated palmitic acid in the presence of avidin. Data are presented for APG (A), MGDG (B), PG (C), DGDG (D), and GPDGDG (E).

Table 2: Fatty Acid Compositions of the Major Membrane Lipids from *A. laidlawii* Cells Grown with Equimolar Exogenous Perdeuterated Palmitic Acid and Non-perdeuterated Elaidic Acid in the Presence of Avidin[a]

	fatty acid composition (mol %)		
membrane lipid	palmitate	elaidate	others
MGDG	54.3 ± 0.2	45.5 ± 0.2	0.2
DGDG	45.6 ± 0.2	54.3 ± 0.2	0.1
PG	44.9 ± 0.3	54.9 ± 0.3	0.2
GPDGDG	47.0 ± 1.1	52.9 ± 1.1	0.1
APG[b]	52.3 ± 0.9	47.5 ± 0.9	0.2

[a] Values presented are the arithmatic means (averages), and average deviations from the means, of three independent determinations of the fatty acid compositions of the same lipid samples used for the DSC, QELS, and NMR analyses presented elsewhere in this paper. [b] The values presented are for the fatty acyl group attached to the 2-position of the glucose polar headgroup; the 1-position of the glucose moiety contains an ether-linked polyprenyl hydrocarbon chain.

range 5−80 °C. Specifically, aqueous dispersions of APG exhibit a lamellar crystalline (L_c) to H_{II} phase transition at about 60 °C [in samples cooled rapidly and immediately heated, this compound also exhibits a lamellar gel (L_β) to liquid-crystalline (L_α) phase transition temperature near 25 °C and an L_α to H_{II} phase transition near 40 °C]. Aqueous dispersions of MGDG and PG exhibit reversible, fairly cooperative, endothermic L_β to L_α phase transitions at temperatures near 40 and 20 °C, respectively; as well, the MGDG exhibits a less energetic but reversible L_α to H_{II} transition at about 55 °C. Aqueous dispersions of DGDG exhibit a broad, more energetic L_c to L_α phase transition at about 40 °C on heating (or a sharper, less energetic L_β to L_α phase transition near 20 °C in rapidly cooled and immediately reheated samples). However, aqueous dispersions of GPDGDG do not exhibit any detectable phase transitions over the temperature range 5−80 °C in the absence of calcium (Figure 1E). Since the fatty acid composition of GPDGDG is similar to that of the other lipids (Table 2), this result provides support for the idea that this lipid, in isolation, does not form, nor undergo transitions between, different lamellar or reversed cubic or hexagonal phases at physiologically relevant temperatures.

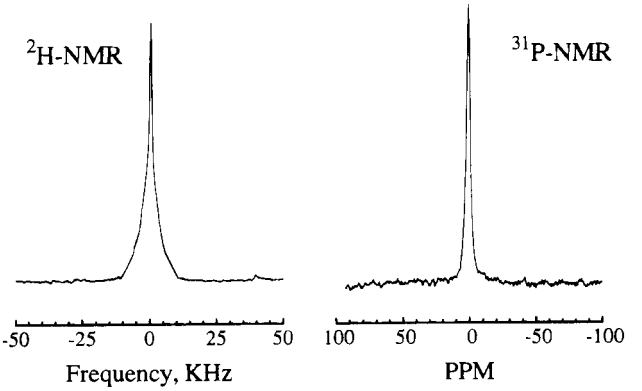

FIGURE 2: ^{2}H- (left panel) and ^{31}P- (right panel) NMR spectra of GPDGDG isolated from membranes of *A. laidlawii* B cells grown in avidin-containing media supplemented with an equimolar mixture of elaidic acid and perdeuterated palmitic acid. The spectra were both acquired at temperatures near 25 °C.

In order to characterize the size and shape of the aggregates formed by GPDGDG in aqueous solution, we employed QELS (data not presented). The QELS analysis indicated that the size distribution of GPDGDG aggregates is clearly bimodal, consisting of two broad, almost nonoverlapping populations. The major population of particles has an effective diameter of 17−19 nm, and the minor population has an effective diameter of 105−115 nm. Static light scattering measurements at various angles of incident light indicated that on average these particles were not spherical but rather possessed an appreciable axial ratio. Since the effective sizes of the small particles are near or below the minimum size (~20−25 nm) of even heavily sonicated, closed lamellar phases (liposomes), we tentatively identified these aggregates as spherical or elliptical micelles. Although the larger particles could in principle be liposomes, their appreciable axial ratio, the absence of a lamellar gel to liquid-crystalline phase transition by DSC (see above), and the absence of a lamellar phase NMR signature (see below) seem to preclude this possibility. Since three-dimensional reversed cubic or hexagonal phases do not scatter light as discrete particles, we tentatively identify these larger aggregates as rod-like micelles. In fact, the presence of spherical and rod-like micelles in aqueous dispersions of GPDGDG has been directly confirmed by cryo transmission electron microscopy (G. Lindbom, L. Rilfors, and Y. Talmon, personal communication).

We also performed ^{2}H- and ^{31}P-NMR spectroscopic studies of aqueous dispersions of GPDGDG to gain additional information about the nature of the aggregates formed by GPDGDG in water. As shown in Figure 2, both NMR spectra consist of single, sharp, symmetric resonances, indicating that the motions of both the perdeuterated palmitoyl chain and the phosphate moiety of the polar headgroup of GPDGDG are rapid and isotropic on their respective NMR time scales. In principle, the isotopic signal could arise either from the presence of a cubic phase, from populations of small liposomes tumbling rapidly on the NMR time scale, or from the presence of normal micelles. However, if the large particles represented lamellar or reversed hexagonal phases, characteristic NMR signatures should have been detected (Cullis et al., 1983; Rilfors et al., 1984; Gruner et al., 1985; Gruner, 1992). The observed NMR spectra are thus consistent with the existence of lipid micelles or reversed cubic phases. However, the relatively large polar headgroup of

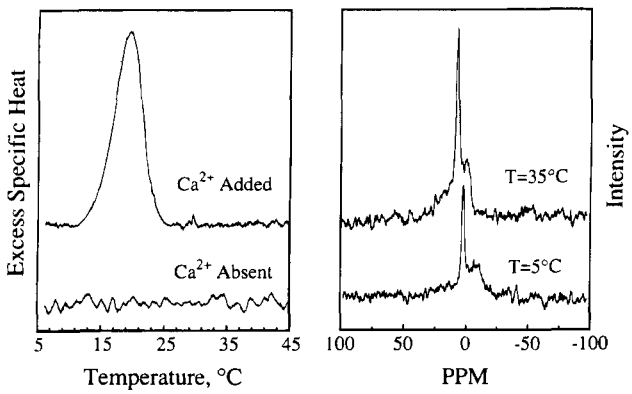

FIGURE 3: Effect of added Ca^{2+} ions on the thermotropic phase behavior of GPDGDG isolated from membranes of *A. laidlawii* B cells grown in perdeuterated palmitic and elaidic acid in the presence of avidin. The left panel shows high-sensitivity DSC thermograms obtained in the presence and absence of 10 mM Ca^{2+}. ^{31}P-NMR spectra obtained in the presence of Ca^{2+} ions are shown in the right panel.

the GPDGDG molecule should not favor the formation of a reversed phase, again supporting the existence of lipid micelles. Moreover, ^{2}H-NMR studies of water diffusion in aqueous dispersions of GPDGDG in $^{2}H_2O$ confirm the micellar nature of this lipid component (G. Lindblom and L. Rilfors, personal communication).

We have two other pieces of evidence that GPDGDG in isolation forms micellar phases. The addition of a 10-fold excess of Ca^{2+} to GPDGDG dispersions results in the development of some opacity and in the induction of a lamellar gel to liquid-crystalline phase transition near 18 °C detectable by high-sensitivity DSC (see Figure 3, left panel). Again, in the absence of Ca^{2+}, no phase transition is detected by this technique. Moreover, Ca^{2+} addition also results in the development of a characteristic lamellar phase NMR signature (see Figure 3, right panel). In the presence of excess Ca^{2+}, ^{31}P-NMR spectra, consisting of a superposition of an isotropic peak and an axially symmetric powder pattern with a high-field peak and a low-field shoulder, are observed at temperatures both above and below the calorimetrically detectable phase transition induced by Ca^{2+} addition. The composite ^{31}P-NMR spectra shown in Figure 3 are consistent with the coexistence of normal micellar and lamellar phases in this sample. Thus, in the presence of excess Ca^{2+}, GPDGDG appears to form a mixture of micellar and lamellar phases, the ratio of which depends on the Ca^{2+} concentration (data not presented). The reason that Ca^{2+} can induce the formation of lamellar phases in GPDGDG may arise from its ability to convert the like-charge repulsion between the negatively charged phosphate moieties of the adjacent GPDGDG headgroups into a net attractive interaction, thus reducing the effective polar headgroup size and converting the GPDGDG molecule from a conical to a more cylindrical shape. The addition of Ca^{2+} may also alter the conformation and reduce the motional freedom of the GPDGDG headgroup and/or reduce its hydration, thereby decreasing its effective cross-sectional area. The finding that the addition of Ca^{2+} can induce formation of the lamellar phase in GPDGDG is important because it confirms that if GPDGDG did form lamellar phases in the absence of divalent cations, the various physical techniques which we have employed would have been able to detect this fact.

Finally, in the preceding paper we have assessed the strengths of the relative phase preferences of all of the major *A. laidlawii* B membrane lipids by determining the effect of the addition of small amounts of the dielaidoyl species of each lipid on the L_α to H_{II} phase transition temperature of a DEPE matrix (Foht et al., 1995, accompanying paper). As expected, MGDG and APG destabilized the L_α phase and stabilized the H_{II} phase while PG, DGDG, and GPDGDG had the opposite effect. Especially noteworthy in the present context, however, is the fact that GPDGDG was considerably more potent on a molar basis in stabilizing the L_α phase and destabilizing the H_{II} of DEPE than are PG or DGDG. In fact, concentrations of GPDGDG in excess of 5 mol % abolished the ability of DEPE to form the H_{II} phase at experimentally accessible temperatures. Significantly, comparable amounts of the gangliosides of eukaryotic cells can also exhibit a similar ability to strongly destabilize the formation of the H_{II} phase in DEPE aqueous dispersions (Perillo et al., 1994), and these compounds also form micelles in isolation (Maggio, 1985; Maggio *et al.*, 1988; Acquotti et al., 1994). Thus, the very large effective size of the GPDGDG polar headgroup, as revealed in the accompanying study, is fully compatible with the ability of this compound to form micelles in isolation.

DISCUSSION

Our finding that GPDGDG spontaneously forms micelles when dispersed in excess water is, to the best of our knowledge, the first demonstration of the existence of a major normal micelle-preferring lipid in a microbial biological membrane. Although it has also been reported that aqueous dispersions of some gangliosides can also form micelles in isolation, these compounds are normally found only in small quantities, primarily on the external surface of the plasma membranes of higher animals. Nevertheless, these glycosphingolipids may have significant effects on the overall phase preference of the lipids in these membranes (Perillo et al., 1994). Although under some circumstances the GPDGDG component may also be present in small amounts, this lipid can make up nearly 30 mol % of the total lipids when supplemented with appropriate exogenous fatty acids, suggesting that the presence of this lipid must have a major effect on membrane lipid phase preference. Although the existence of GPDGDG in a variety of Gram-positive eubacteria has long been known (O'Leary & Wilkinson, 1988), the phase preference of this lipid had not previously been established. Whether or not significant amounts of other normal phase-preferring lipids exist in the membranes of other prokaryotic microorganisms is presently an open question. However, at the very least the present work establishes that not all of the lipid components present in all biological membranes prefer to form lamellar or reversed phases when dispersed in water, as is commonly assumed.

The question can be posed as to whether or not the characteristic alterations in GPDGDG levels, in response to variations in membrane lipid fatty acid composition, represent a coherent regulatory response by this organism which effectively maintains an optimal bilayer/nonbilayer phase balance of its membrane lipids. Since the relative amount of GPDGDG present progressively increases as membranes are enriched in palmitate, elaidic, oleic, and linoleic acids (see Table 2), and since there is an increased tendency for the MGDG component of these membranes to form a

reversed phase as the degree of unsaturation increases, one could argue that these progressively elevated GPDGDG levels represent a compensatory biochemical mechanism to counteract this tendency. However, even higher GPDGDG levels are noted when membrane lipids are enriched in the methyl isobranched fatty acids, and GPDGDG levels *decrease* with increases in chain length in this series of fatty acids. Since the tendency of MGDG to form reversed phases is much less pronounced with methyl isobranched than with *cis*-unsaturated fatty acids of comparable length, and since this tendency increases with *increases* in hydrocarbon chain length, the observed variations would not be predicted in this case. Also, it is puzzling that biosynthetic enrichment in, for example, methyl anteisobranched fatty acids, which are fairly potent promoters of the tendency of MGDG to form reversed phases, fails to elevate GPDGDG levels. Thus, it does not appear that *A. laidlawii* B utilizes variations in GPDGDG levels to consistently regulate the phase preference of its membrane lipids, although clearly, additional biophysical studies of the thermotropic phase behavior of the total membrane lipids of this organism will be required to confirm this conclusion. In this regard, it may be noteworthy that elevations in GPDGDG levels seem to be coupled with decreases in the levels of DGDG, its presumed biochemical precursor (Dahlqvist et al., 1992; McElhaney, 1992). It is thus possible that variations in fatty acid composition affect the rate of formation of GPDGDG from DGDG because of the molecular species substrate specificity of the relevant enzyme (or enzymes) and that this substrate specificity may not be related in any direct way to the bilayer/nonbilayer phase promoting tendencies of these fatty acids. Otherwise, it is difficult to rationalize the observation that GPDGDG levels are elevated under conditions which should both promote (incorporation of long chain *cis*-unsaturated fatty acids) and retard (incorporation of short chain methyl isobranched fatty acids) reversed lipid phase formation in the membrane of this organism.

The lipid polar headgroup compositional data presented in Table 1 also illustrate another important point, related to the use of the MGDG/DGDG ratio as an indicator of the relative bilayer/nonbilayer phase preference of the *A. laidlawii* membrane lipids. It is clear from our data that this ratio, which has been used in all previous studies of lipid biosynthetic regulation in both the A and B strains, is *not* a reliable indicator of the relative amounts of bilayer and nonbilayer lipids, or of the relative potencies of the nonbilayer lipid components, at least in the membrane of *A. laidlawii* B. For example, membranes enriched in exogenous palmitic acid have the lowest MGDG/DGDG ratio of the membranes studied here, which would seem to imply that they also have the smallest quantity of reversed phase-preferring lipid. However, this is clearly not the case, since in fact these membranes contain the largest amount of H_{II}-preferring lipids (5.3 mol % MGDG plus 69.3 mol % APG). The decreased MGDG/DGDG ratio observed is in a sense an artifact, arising from the fact that the elevated levels of AGP occur primarily at the expense of the MGDG component. Moreover, based on the relative lamellar/H_{II} phase transition temperatures of the pure lipids and on their relative effects on the L_α/H_{II} phase transition temperature of a phosphatidylethanolamine matrix (Foht et al., 1995, accompanying paper), the APG is a more potent H_{II} phase-forming lipid than the MGDG which it largely replaces (Lewis et al., 1990). Similarly, the decreasing MGDG/DGDG ratios observed as the chain length of the biosynthetically incorporated methyl isobranched fatty acid increases would seem to imply a decreasing amount of reversed phase-preferring lipid in these membranes. However, this is clearly not so, since the absolute and relative amount of MGDG present changes relatively little with the chain length of the biosynthetically incorporated branched-chain fatty acid. Moreover, the presence of the appreciable and progressively decreasing amounts of the micelle-preferring GPDGDG component, which must affect the overall lamellar/nonlamellar phase preference of the total membrane lipids considerably (Foht et al., 1995), is not accounted for at all in such an analysis. Clearly, in future studies of lipid polar headgroup biosynthetic regulation in this organism, a more reliable and comprehensive index of bilayer/nonbilayer phase preference is required. This index must take into account all of the lipid species present in the membrane, including those components which prefer to form micelles in isolation, and must also consider the relative strengths of the characteristic phase preferences of the individual lipid components. Other considerations, such as the possible transverse lipid asymmetry of the individual membrane lipids, or their preferential interaction with membrane proteins, may also be relevant. Such an analysis is currently being carried out in this laboratory using *A. laidlawii* B membranes from cells biosynthetically enriched with a wide variety of different types of exogenous fatty acid of varying hydrocarbon chain lengths.

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