Key Role of the Diglucosyldiacylglycerol Synthase for the Nonbilayer−Bilayer Lipid Balance of *Acholeplasma laidlawii* Membranes†

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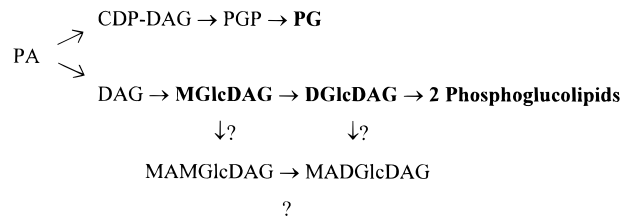
ABSTRACT: In the single membrane of *Acholeplasma laidlawii*, a specific glucosyltransferase (DGlcDAG synthase) synthesizes the major, bilayer-forming lipid diglucosyldiacylglycerol (DGlcDAG) from the preceding major, nonbilayer-prone monoglucosyldiacylglycerol (MGlcDAG). This is crucial for the maintenance of phase equilibria close to a potential bilayer−nonbilayer transition and a nearly constant spontaneous curvature for the membrane bilayer lipid mixture. The glucolipid pathway is also balanced against the phosphatidylglycerol (PG) pathway to maintain a certain lipid surface charge density. The DGlcDAG synthase was purified ∼5000-fold by three chromatographic techniques and identified as a minor 40 kDa membrane protein. In CHAPS mixed micelles, a cooperative dependence on anionic lipid activators was confirmed, with PG as the best. The dependence of the enzyme on the soluble UDP-glucose substrate followed Michaelis−Menten kinetics, while the kinetics for the other (lipid) substrate MGlcDAG exhibited cooperativity, with Hill coefficients in the range of 3−5. V_{max} and the Hill coefficient, but not K_m, for the MGlcDAG substrate were increased by increased PG concentrations, but above 3 mol % MGlcDAG, the rate of synthesis was constant. Hence, the DGlcDAG synthase is more affected by the lipid activator than by the lipid substrate at physiological lipid concentrations. The enzyme was shown to be sensitive to curvature "stress" changes, i.e., was stimulated by various nonbilayer lipids but inhibited by certain others. Certain phosphates were also stimulatory. With the two purified MGlcDAG and DGlcDAG synthases reconstituted together in the presence of a potent nonbilayer lipid, the strong responses in the amounts of MGlcDAG and DGlcDAG synthesized mimicked the responses in vivo. This supports the important regulatory functions of these enzymes.

Properties of membrane lipids are important for both the structure and function of membrane proteins. In the single membrane of the cell wall-less bacterium *Acholeplasma laidlawii*, a specific enzyme (DGlcDAG[1] synthase) synthesizes the major, bilayer-forming lipid diglucosyldiacylglycerol (DGlcDAG) from the major, nonbilayer-prone monoglucosyldiacylglycerol (MGlcDAG). This is crucial for the maintenance of phase equilibria close to a bilayer−nonbilayer transition and a nearly constant spontaneous curvature in the membrane lipid bilayer, which is achieved by extensive variations in the polar lipid composition as a response to external or internal factors affecting the physical properties of the lipid bilayer. The DGlcDAG synthase catalyzes the transfer of glucose from UDP-glucose to MGlcDAG to yield DGlcDAG (and UDP), and is therefore a glucosyl transferase. This membrane-bound enzyme, 1,2-diacylglycerol-3-α-glucose-(1→2)-α-glucosyl transferase (DGlcDAG synthase), is purified to almost homogeneity and characterized in this work.

The polar lipids in *A. laidlawii* are all made from a minor phosphatidic acid (PA) precursor in two competing pathways: one leading to the major (in bold below) phosphatide phosphatidylglycerol (PG) and the other by way of the minor 1,2-diacylglycerol (DAG) leading sequentially to the major glucolipids MGlcDAG and DGlcDAG. DGlcDAG is probably also the precursor for two (usually minor) phosphoglucolipids.



Key lipids in *A. laidlawii* for regulation of the packing properties are MGlcDAG and DGlcDAG, forming mainly nonlamellar and lamellar phases, respectively (*1*). Via

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[1] Abbreviations: 1,2-DAG, 1,2-diacylglycerol; DGlcDAG, 1,2-diacyl-3-*O*-[α-D-glucopyranosyl(1→2)-*O*-α-D-glucopyranosyl]-*sn*-glycerol; DO, dioleyl; 1,3-DOG, 1,3-dioleoylglycerol; DPG, diphosphatidylglycerol; Glc, glucose; MADGlcDAG, 1,2-diacyl-3-*O*-[α-D-glucopyranosyl(1→2)-*O*-(6-*O*-acyl-α-D-glucopyranosyl)]-*sn*-glycerol; MAMGlcDAG, 1,2-diacyl-3-*O*-[6-*O*-acyl(α-D-glucopyranosyl)]-*sn*-glycerol; MGalDAG, 1,2-diacyl-3-*O*-(β-D-galactopyranosyl)-*sn*-glycerol; MGlcDAG, 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PGD, dodecylphosphoglycerol; PS, phosphatidylserine; Sph, sphingosine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; IEC, ion exchange chromatography; HAC, hydroxyapatite chromatography; DLAC, dye ligand affinity chromatography; TLC, thin layer chromatography.

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metabolic alteration of the proportions of MGlcDAG and DGlcDAG, the phase equilibria and spontaneous curvature are maintained in the membrane by the cells. Under certain conditions, when MGlcDAG becomes more bilayer-prone, more potent nonbilayer variants of the two glucolipids with an extra, third acyl chain (i.e., MAMGlcDAG and MADGlcDAG) (*2*, *3*) are also synthesized (*4*, *5*). In vivo, the syntheses of the major bilayer and nonbilayer membrane glucolipids are strongly affected by the acyl chain length and extent of unsaturation in the bilayer matrix, and by the presence of foreign, amphiphilic, or hydrophobic perturbant molecules in the membrane (*4*, *6*). With crude enzymes in vitro, the syntheses of MGlcDAG and DGlcDAG are dependent upon the presence of a certain surface charge density of anionic phospholipids, and upon critical and substantial amounts of an activator lipid [1,2-dioleoylphosphatidylglycerol (DOPG)], respectively. The MGlcDAG synthase is probably the main site for a lipid surface charge regulation, balancing the two pathways (*7*). On the other hand, the activity of the DGlcDAG synthase is very sensitive to the type of bilayer matrix and is stimulated by nonbilayer-forming molecules, most likely as a response to changes in the spontaneous curvature (*8*). Hence, the bilayer−nonbilayer balance and curvature are more likely regulated by the DGlcDAG synthase. This enzyme is also stimulated by a domain formation of the activator PG, caused by a chain length mismatch (*9*). Furthermore, there are indications that the MGlcDAG−DGlcDAG metabolic balance is connected to the energy status of *A. laidlawii* (*10*, *11*). The variations in activity of this enzyme with different properties of the amphiphilic and aqueous environment, with no other cytoplasmic or membrane proteins present, are therefore particularly interesting with respect to the understanding of the bilayer spontaneous curvature in *A. laidlawii*.

We report here the purification and characterization of the DGlcDAG-synthesizing enzyme 1,2-diacylglycerol-3-α-glucose(1→2)-α-glucosyl transferase from *A. laidlawii*. The sensitivity and responsiveness of this membrane-bound enzyme to various types of amphiphiles, in addition to phosphates in the aqueous surroundings, suggest that it is a key step for an integrated regulation of bilayer packing properties in the cell.

MATERIALS AND METHODS

Strain and Growth Conditions. A. laidlawii strain A-EF22 was cultivated in an oleic acid (18:1c)-supplemented medium and harvested, and the protein content of cells and membranes was determined, as described by Dahlqvist et al. (*8*).

Lipids Used in the Experiments. Synthetic *rac*-1,2-diacylglycerol (1,2-DOG) and *rac*-1,3-diacylglycerol (1,3-DOG) were purchased from Larodan (Malmö, Sweden). 1,2-Dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoylphosphatidylcholine (DOPC), 1,2-dioleoylphosphatidylserine (DOPS), 1,2-dioleoylphosphatidylethanolamine (DOPE), and diphosphatidylglycerol (DPG) were from Avanti Polar Lipids (Alabaster, AL). Bovine brain D-sphingosine (Sph) and cholest-4-en-3-one were purchased from Sigma (St. Louis, MO). The MGlcDAG and DGlcDAG were prepared from *A. laidlawii* cells grown in the presence of oleic acid, which gives >90% (moles to mole) 18:1c lipid acyl chains (*8*). Synthetic, saturated MGlcDAGs and MGalDAGs were kindly donated by D. Mannock (Department of Biochemistry, University of Alberta at Edmonton, Edmonton, AB). Plant MGalDAG was kindly provided by E. Selstam (Department of Plant Physiology, Umeå University). Glucolipid GP derivatives and MAMGlcDAG and MADGlcDAG from *A. laidlawii* were kindly provided by A.-S. Andersson (Department of Physical Chemistry, Umeå University). The detergent CHAPS was from Boehringer Mannheim, and dodecylphosphoglycerol (PGD) was from Alexis.

Cell Solubilization. Harvested cell suspensions (∼25 mg of cell protein/mL) were mixed with a solubilization buffer [24 mM Tris maleate (pH 8), 30 mM CHAPS, and 20% (v/v) glycerol] to a concentration of ∼3 mg of protein/mL (*12*). After 60 min on ice, with gentle swirling of the mixture every 15 min, an insoluble fraction containing no significant DGlcDAG synthesis activity was removed by centrifugation at 20000*g* for 30 min at 2 °C. During the entire purification procedure, low temperatures (<4 °C) and a high concentration of glycerol [20% (v/v)] were conditions required for purification of an active enzyme.

Ion Exchange Chromatography (IEC). The NaCl concentration in the solubilized protein preparation was raised to 0.4 M by adding a 4 M NaCl solution. Glycerol was also added to maintain a level of 20% (v/v). A column (50 mm in diameter) packed with 90 mL of SP-Sepharose (Pharmacia) was equilibrated with at least 500 mL of 20 mM NaH_2PO_4/Na_2HPO_4 (pH 8), followed by 450 mL of 20 mM NaH_2PO_4/Na_2HPO_4 (pH 8), 20% (v/v) glycerol, and 0.4 M NaCl, and finally 135 mL of IEC buffer [20 mM NaH_2PO_4/Na_2HPO_4 (pH 8), 20% (v/v) glycerol, 20 mM CHAPS, and 0.4 M NaCl]. Then the solubilized cells were loaded, and the column was washed with another 243 mL of IEC buffer. The DGlcDAG-synthesizing enzyme was eluted with a 250 mL gradient of 0.4 to 1.0 M NaCl in IEC buffer. Last, the remaining proteins were eluted with 120 mL of 1.0 M NaCl in IEC buffer. Fractions (10 mL) were collected during the chromatography, analyzed for protein concentration and enzyme activity, and stored at −80 °C. Endogenous lipids were most likely removed from the proteins by the excess of detergent present during the procedure. No lipid determination was performed, but the yellow color that is very characteristic of *A. laidlawii* lipid extracts was washed out of the column before the enzyme.

Hydroxyapatite Chromatography (HAC). A column (16 mm in diameter) packed with 6 mL of ceramic hydroxyapatite Macro-Prep (Bio-Rad) was equilibrated with HAC buffer [100 mM KH_2PO_4/K_2HPO_4 (pH 8), 20% (v/v) glycerol, and 20 mM CHAPS]. Fractions eluted after the major protein peak in the IEC (above) were pooled and diluted two times with dilution buffer [20 mM KH_2PO_4/K_2HPO_4 (pH 8), 20% (v/v) glycerol, and 20 mM CHAPS]. This sample was loaded onto the HAC column. After the column had been washed with 24 mL of HAC buffer, the enzyme was eluted with 51 mL of HAC buffer containing a gradient of 200 to 408 mM KH_2PO_4/K_2HPO_4. Proteins remaining in the column were then eluted with 38 mL of 465 mM KH_2PO_4/K_2HPO_4 in HAC buffer. Fractions (2.5 mL) were collected and analyzed with respect to protein content and enzyme activity.

Dye Ligand Affinity Chromatography (DLAC). A column (10 mm in diameter) packed with 10 mL of Green A agarose gel (Amicon) was equilibrated with DLAC buffer [20 mM

HEPES (pH 8), 20% (v/v) glycerol, and 20 mM CHAPS]. Enzyme fractions eluted from two HAC columns (above) were pooled and diluted two and one-half times according to the salt concentration; dilution was achieved with 20 mM HEPES (pH 8), 20% (v/v) glycerol, and 20 mM CHAPS. The sample was then loaded onto the DLAC column. After the column had been washed with 30 mL of DLAC buffer, the enzyme was eluted with 60 mL of DLAC buffer containing a gradient of 0.4 to 1.9 M KCl. Proteins remaining in the column were eluted with 30 mL of 2.5 M KCl in DLAC buffer. Fractions (3 mL) were collected, analyzed with respect to protein content and enzyme activity, and stored at −80 °C.

All solutions used throughout the chromatography steps were temperature equilibrated at 4 °C, and all the procedures were performed at 4 °C. The BCA kit (Pierce) was used for protein determination. The protein composition in the various fractions was analyzed with SDS−polyacrylamide gel electrophoresis, as described by Nyström et al. (*13*). The N-terminal amino acid sequence for the DGlcDAG synthase (40 kDa band in Figure 2), as well as the 30 kDa band, was analyzed by the Edman degradation method performed by B. Ek (Department of Plant Biology, Swedish University of Agricultural Sciences, Uppsala, Sweden). In addition, we also tried various affinity media (blue, red, and orange dye ligand chromatography and three types of UDP-agarose) for purification of the enzyme, but all without success.

Assay for DGlcDAG Synthase Activity. Mixed micellar dispersions were prepared by mixing lipids solubilized in 1,1,1-trichloroethane or chloroform/methanol (2/1 v/v) to the required concentrations. The solvent was evaporated under a stream of N_2. Lipid mixtures were then solubilized to homogeneity in a CHAPS assay buffer [100 mM Tris maleate (pH 8), 20 mM $MgCl_2$, and 19 mM CHAPS] by extensive vortexing, followed by overnight incubation at 4 °C, and bath sonication for 5 min. In the standard assay, 10 μL of enzyme solution (237 ng of protein) was added to 80 μL of micellar solution. The mixtures were kept for 25 min on ice and preincubated for 5 min at 28 °C. Reactions were started by addition of 10 μL of UDP-[^{14}C]glucose to give a concentration of 1 mM in a volume of 100 μL in assay buffer. The standard lipid concentration was 11 mM (0.3−0.75 mM MGlcDAG substrate, with activator DOPG and matrix lipids). Fractions from chromatography columns were analyzed in 50 μL samples mixed with 40 μL of micellar solution to give final concentrations of 1 mM MGlcDAG, 9 mM DOPG, 110 mM Tris maleate (pH 8), 20 mM $MgCl_2$, and 20 mM CHAPS, except for the IEC step where 8 mM DOPG was used. The micellar solution preparation and assay were the same as described above. After incubation for 5 min (or 30 min for chromatography fractions) at 28 °C, reactions were stopped with 375 μL of methanol/chloroform (2/1, v/v), and the lipids, including newly synthesized DGlcDAG, were extracted and separated with TLC plates as described by Karlsson et al. (*12*) and then quantified using electronic autoradiography (Packard Instant Imager).

RESULTS

Enzyme Purification and Reconstitution in Mixed Micelles. Purification of the membrane-bound DGlcDAG synthase was started by total solubilization of intact cells in 30 mM CHAPS (a zwitterionic detergent) by the method optimized by Karlsson et al. (*7*, *12*) for the preceding MGlcDAG synthase. Insoluble components were removed by centrifugation, and the micelle solution was loaded directly onto an IEC column (cationic exchange, SP-Sepharose). The DGlcDAG synthase did bind and was separated from the other major glucolipid-synthesizing enzyme MGlcDAG synthase by elution with a concentration gradient of NaCl (0.4 to 1.0 M) (Figure 1A), giving a 190-fold enrichment (Table 1). The activity analyses shown in Table 1 were strongly affected by the presence of phosphate, which stimulated the DGlcDAG activity, and also affected by KCl which inhibited the activity. Phosphate or KCl was present during the assays depending on the chromatography technique that was used (see Materials and Methods). The DGlcDAG synthase from the previous IEC step did bind to a ceramic hydroxyapatite matrix at pH 8 and was essentially separated from the major peak of retained proteins by elution with a concentration gradient of KH_2PO_4/K_2HPO_4. The DGlcDAG synthase was eluted at ∼300 mM phosphate (Figure 1B), giving a total enrichment factor of 100. Because of dilution, and since not all of the best fractions were used in the following purification step, it is likely that the actual enrichment factor of the enzyme by the hydroxyapatite was higher. The final step in the purification procedure was dye ligand affinity chroma-

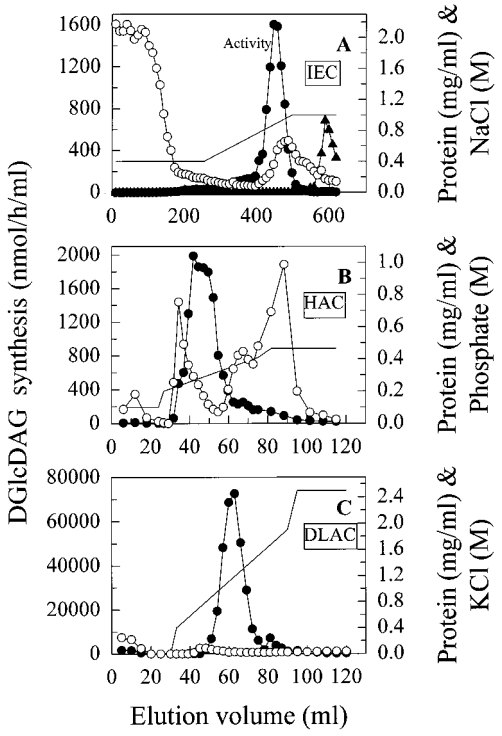

FIGURE 1: Purification of the DGlcDAG synthase by three column chromatography techniques. Protein (○) and DGlcDAG synthesis activity were eluted in three different, consecutive chromatographic steps as described in Materials and Methods. (A) Ion exchange chromatography on SP-Sepharose (pH 8). MGlcDAG synthesis activity (▲) was eluted differently from DGlcDAG synthesis activity. The solid line without symbols is the applied NaCl concentration. (B) Hydroxyapatite chromatography on ceramic hydroxyapatite Macro-Prep (pH 8). The solid line without symbols is the applied KH_2PO_4/K_2HPO_4 concentration. (C) Dye ligand affinity chromatography on Green A (pH 8). The solid line without symbols is the applied KCl concentration. The assay for DGlcDAG synthesis and protein determination was performed as described in Materials and Methods.

Table 1: Purification of the Monoglucosyldiacylglycerol Glucosyltransferase from Cells of *A. laidlawii*[a]

purification step	protein[b] (μg)	total activity[b] (nmol/h)	specific activity (nmol mg^{-1} h^{-1})	yield in step	total yield	enrichment factor in step (%)	total enrichment factor
cell solubilization	7171000	66296	9				
ion exchange chromatography[c]	246600	435200[f]	1764	656	656	191	191
hydroxyapatite chromatography[d]	33644	30949	919	7.1[f]	46[f]	0.5[f]	100
Dyematrix[e]	71	3432	48306	11	5.2	525[f]	5250

[a] Cell solubilization, column chromatography, and assays for the enzyme activity and protein concentration were performed as described in Materials and Methods. [b] Figures for protein amounts and enzyme activity at each step are the amounts used in the next step. [c] Figures are the sum of three separate and identical rounds of IEC. [d] Figures are the sum of two separate and identical rounds of HAC. [e] Figures are from DLAC, and not all of the best fractions from HAC were used. [f] Stimulation by phosphate or inhibition by KCl.

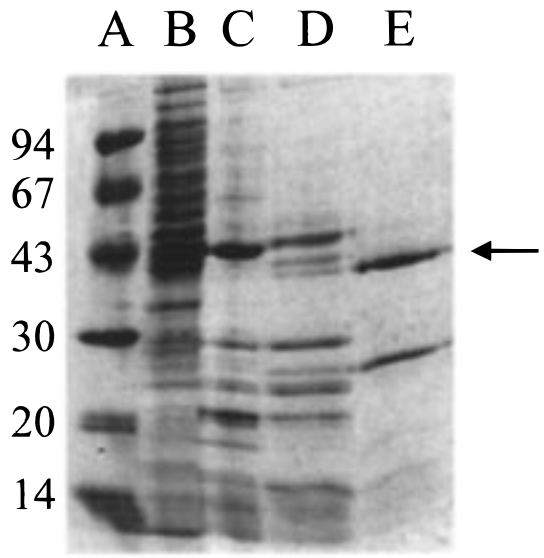

FIGURE 2: SDS−polyacrylamide gel electrophoresis of purified proteins. Selected samples from the purification procedures stained with Coomassie Brilliant Blue: lane A, commercial reference mixture, with molecular masses in kilodaltons; lane B, solubilized whole-cell extract; lane C, pooled fractions after IEC; lane D, pooled fractions after HAC; and lane E, high-activity DLAC fraction. Gels were run (in a Bio-Rad MiniProtean II apparatus), processed, and stained as described by Nyström et al. (*13*).

tography (Dyematrix, Green A). The enzyme was bound to the Green A matrix at pH 8, and eluted from the column in a sharp peak with a KCl gradient (Figure 1C). The enrichment factor of ~500 (Table 1) is probably too high according to the preceding step, but the total enrichment factor of ≈5200 is correct since the initial measurement of activity (after solubilization) was not influenced by ions. In earlier purification rounds, other types of affinity chromatography were used instead of the Dyematrix. The catalytic properties were exploited, and the DGlcDAG synthase was specifically bound to UDP-glucoronic acid agarose in the presence of Mg^{2+} (required for activity) and was eluted with a gradient of KCl, notably enriched.

SDS−polyacrylamide gel electrophoresis of proteins in the eluted high-activity fractions revealed the occurrence of a protein at ≈40 kDa, the amounts of which correlated with the elution profile of the DGlcDAG synthesis activity in the last purification step (as in Figure 1C). This SDS−PAGE pattern, where the DGlcDAG synthesis activity correlated with the 40 kDa protein band, was also found in previous purification preparations with the substrate affinity chromatography technique. The gel photo (Figure 2) shows the DGlcDAG synthase from a high-activity DLAC fraction; the protein band located at 30 kDa was tentatively identified as the RNA-binding protein S4 from the small ribosome subunit, according to its N-terminal amino acid sequence (25 cycles) and molecular mass. The N-terminal amino acid sequence of the purified DGlcDAG synthase (30 cycles) was verified from two different preparations (data not shown), and was completely different from that of the 40 kDa MGlcDAG synthase (*7*).

Before characterization of the DGlcDAG synthase, some important assay conditions were analyzed. The enzyme could be reconstituted, with maintained activity, in liposome bilayers by the dialysis technique (data not shown). However, in mixed, detergent/lipid (bilayer-like) micelles formed by CHAPS (or CHAPSO), no topology constraints occur as in liposome bilayer models. These detergent/lipid aggregates also allow the efficient folding and function of integral and peripheral membrane proteins, and the influence of polar lipids in these processes (*14*−*16*). Furthermore, formation of the mixed aggregates takes place substantially faster than with the dialysis technique, thus better preventing loss of activity. First, the effect on enzyme activity of varying the DOPG/CHAPS ratio was analyzed at different total amphiphile concentrations. A larger DOPG molar fraction was needed for maximal activity at higher total amphiphile concentrations than at lower ones. It could be concluded that the enzyme activity depends on both the amphiphile concentration and the fraction of DOPG, similar to the case with the MGlcDAG synthase (*7*). At CHAPS/lipid molar ratios of >1.04, only mixed micelles (and no lipid bilayers) are formed (*15*). A CHAPS/lipid ratio of ~1.7 was set as a standard to be safely above this transition and to give experimental space for variation in the ratio "effector lipids"/needed lipids (substrate, activator). The total amphiphile concentration was set at 30 mM which is substantially higher than the CHAPS monomer concentration (cmc of 4−12 mM depending on conditions) (*17*). However, the concentration of free detergent in the aqueous phase is substantially lowered by the presence of membrane lipids in the aggregates (*15*, *17*). Second, both different buffers and assay times were analyzed. Tris maleate promoted higher DGlcDAG synthesis activity then HEPES, and the rate of synthesis was constant over time from 5 to 45 min.

Activation by Anionic Lipids. Which polar lipids support the DGlcDAG synthase activity? Using the standard conditions (above) except for the assay time (here 30 min), the proportions of DOPG to other polar lipids in the CHAPS mixed micelles were systematically varied. With the endogenous, uncharged glucolipid DGlcDAG as the matrix lipid, the fraction of DOPG versus other polar lipids had to be >0.7 before any activity was detectable, and then with a slightly sigmoidal appearance (Figure 3). The zwitterionic

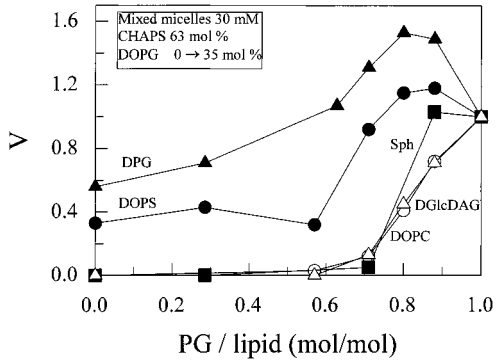

FIGURE 3: Dependence of the DGlcDAG synthase on anionic lipids. Different anionic lipids were stepwise replaced by DOPG. Curves are normalized relative to their common point at a DOPG fraction of 1 (35 mol %).

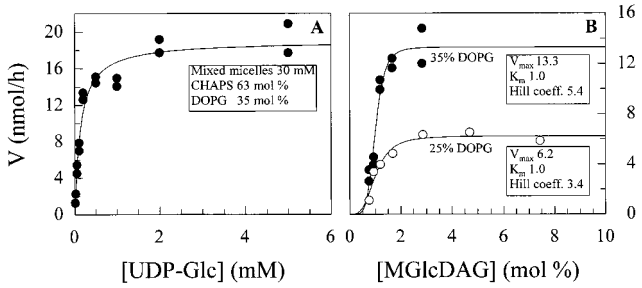

FIGURE 4: Variation of DGlcDAG synthase activity with the concentration of its two substrates. Enzyme activity was analyzed as a function of the (A) UDP-Glc and (B) MGlcDAG concentrations in mixed micelles. The curve in panel A was calculated from the Michaelis−Menten equation fitted to the data points; parameters V_{max} and K_m were determined to be 19 nmol/h and 0.14 mM, respectively. Curves in panel B were calculated with the Hill equation, showing cooperative kinetics.

phospholipid DOPC as the matrix lipid yielded results essentially the same as those for DGlcDAG; this was also valid with the cationic lipid sphingosine (Figure 3). The anionic phospholipid DOPS was able to activate the enzyme, but only to ~33% compared to DOPG. When DOPG and DOPS were mixed in ratios between 0.8 and 0.9, an activity maximum appeared, which indicates that it is a particular physical property (in addition to lipid structural specificity) that leads to maximal activation of the enzyme. The anionic phospholipid bovine diphosphatidylglycerol (DPG) was able to activate the enzyme to ~50% compared to DOPG (Figure 3). When DOPG and DPG were mixed in ratios between 0.7 and 0.9, an activity maximum appeared. Since DPG exhibits large structural similarities in the head region to DOPG and carries two negative charges per molecule, it is not surprising that it had an ability to activate the enzyme also at low concentrations. The anionic, endogenous phosphoglucolipids GPDGlcDAG and MABGPDGlcDAG (here enriched in oleoyl chains), which are glycerophosphoryl derivatives of DGlcDAG (*18*), had no activating effects at all on the enzyme (data not shown). Another potential activator was also examined, the PG-like detergent dodecylphosphoglycerol (PGD), which could not activate the enzyme (cf. Figure 6B below). Hence, the DGlcDAG synthase needs a polar activator lipid, preferably PG, at concentrations of 25−30 mol % (or more) in the zwitterionic, mixed CHAPS bilayer-like aggregate. The PG demand of the purified DGlcDAG synthase seems more selective than for the preceding MGlcDAG synthase (*7*).

Kinetic Characterization of the DGlcDAG Synthase. The DGlcDAG synthase is a bireactant system; the two substrates involved are UDP-Glc and MGlcDAG. For UDP-Glc, the activity of the enzyme correlated in a hyperbolic manner with substrate concentration, and a sharp rise in activity up to 0.5 mM UDP-Glc was evident (Figure 4A). The data could be fitted to the Michaelis−Menten equation with high accuracy. The enzymatic parameters V_{max} and K_m were calculated to be 19 nmol/h and 0.14 mM, respectively. For the other substrate (MGlcDAG), the concentration that was used, i.e., 2.5 mol % (Figure 4A), is close to the V_{max} (see below). Hence, the enzyme was saturated with MGlcDAG in Figure 4A, and the calculated V_{max} for UDP-Glc is valid as the absolute V_{max}. It can be concluded that the DGlcDAG synthase follows Michaelis−Menten kinetics in its dependence on the soluble substrate UDP-Glc.

For the other substrate MGlcDAG, the experimental conditions were more complicated since several parameters had to be simultaneously altered. To keep the total amphiphile concentration constant, the activator/matrix lipid or the CHAPS concentration had to be lowered, both of which may influence the activity and alter the micellar structure (data not shown). Enzyme activity correlated with the MGlcDAG substrate concentration in a sigmoidal manner when the MGlcDAG concentration was altered from 0.75 to 2.8 mol % (35 mol % DOPG) or from 0.85 to 5.3 mol % (25 mol % DOPG) (Figure 4B). MGlcDAG concentrations between 0.75 and 2.8 mol % only shifted the total amphiphile concentration from 31.1 to 31.8 mM, with smaller effects on activity. The data could not be fitted to the Michaelis−Menten equation. However, the data could be fitted to the Hill equation with high accuracy, and the enzymatic parameters V_{max} and K_m could be estimated (Figure 4B). The Hill coefficients at 35 mol % DOPG and 25 mol % DOPG were calculated to 5.4 and 3.4, respectively. This indicates an increase in cooperativity with increasing DOPG concentrations. The dependence of the enzyme activity on MGlcDAG concentration basically followed the same pattern at the two DOPG concentrations, and concentrations of MGlcDAG above 6 mol % had no influence on the activity. In an unsaturated membrane in vivo, the concentration of MGlcDAG usually varies between 10 and 30 mol %. Hence, the DGlcDAG synthase is more affected by the lipid activator DOPG than its lipid substrate MGlcDAG at physiological concentrations of the latter. Calculated values of V_{max} revealed that the catalytic rate of the enzyme was altered by the variation in DOPG concentration but that the K_m remained the same. The latter indicates that DOPG does not influence the enzyme activity by altering the interaction between the enzyme and the MGlcDAG substrate, but is affecting the turnover number of the enzyme reaction. This confirms the data from crude enzyme experiments (*8*), and extends the importance of the activator lipid PG, "controlling" the activity of the DGlcDAG synthase.

Different Lipid Substrates. Synthetic species with sugar headgroup α- or β-isomers of various potential lipid substrates, i.e., MGlcDAG or MGalDAG containing saturated acyl or alkyl chains with similar chain lengths (16:0 or 17:0), were employed to determine the substrate specificity (Table 2). The substrate that gave the highest DGlcDAG

Table 2: Different Lipid Substrates for the DGlcDAG Synthase[a]

lipid substrate	formation of disugar-DAG[b]	relative activity[c] (%)
di-18:1c-α-MGlcDAG	+	100
di-17:0-α-MGlcDAG	+	73
di-17:0-β-MGlcDAG	−	ND[d]
di-16:0-α-MGalDAG	−	ND
di-17:0-β-MGalDAG	−	ND
MA-α-MGlcDAG	−	ND

[a] The assays were performed as described in Materials and Methods. The lipid substrate concentration was 2.5 mol %. [b] Analyzed (TLC) as the formation of radioactive disugar-DAG from substrate and UDP-[^{14}C]glucose. [c] The values have been normalized to the enzyme activity where the lipid substrate was the native di-18:1c-α-MGlcDAG. One hundred percent activity corresponds to 13.3 nmol of DGlcDAG/h. [d] ND, no product detected.

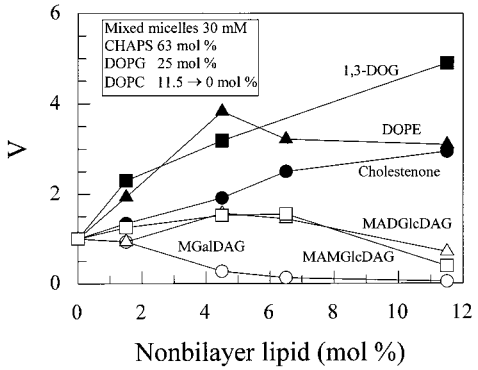

FIGURE 5: Dependence of the DGlcDAG synthase on nonbilayer lipids. Different lipids, with established nonbilayer potencies, stepwise replaced DOPC as a matrix lipid (at a fixed CHAPS concentration). Curves are normalized relative to their common point at a DOPC fraction of 1 (11.5 mol %).

activity was native di-18:1c-α-MGlcDAG; the di-17:0-α-MGlcDAG yielded substantially less activity. In earlier studies with the crude enzyme in a vesicular system, the optimal substrate chain length was 15 carbons (*8*). α-MGalDAG as the lipid substrate could not be processed to GlcGalDAG, indicating that the position of the hydroxyl group at C-4 is important for activity, perhaps for binding of the substrate to the active site of the enzyme. No activity was obtained when the sugar was in the β-position for any of the MGlcDAG or MGalDAG substrate lipids. With a more acylated MGlcDAG, i.e., MA-α-MGlcDAG, as a matrix lipid the DGlcDAG synthase activity remained the same up to 6 mol % MA-α-MGlcDAG (Figure 5); at this point, the DGlcDAG synthase activity started to decrease. MA-α-DGlcDAG as the matrix lipid yielded results that were essentially the same as those for MA-α-MGlcDAG (Figure 5). Hence, these two lipids are therefore probably inhibitors of the DGlcDAG synthase. The unsaturated β-MGalDAG lipid from chloroplasts as the matrix lipid yielded a faster decrease in activity than the *A. laidlawii* MA-glucolipid variants (Figure 5), and seems therefore to be a more effective inhibitor. Calculated values of V_{max} and K_m for MGlcDAG revealed that the catalytic rate of the enzyme was altered by the presence of MAMGlcDAG, but that K_m remained essentially the same. From this, it may be assumed that MAMGlcDAG (and probably also MADGlcDAG) is a noncompetitive inhibitor. Chloroplast β-MGalDAG might be a competitive inhibitor because it inhibited the activity of the DGlcDAG synthase much more efficiently, cf. Figure 5 (up to 99%, data not shown).

Effects of Nonbilayer Lipids. The balance between MGlcDAG (nonbilayer-prone) and DGlcDAG (bilayer-forming) amounts in *A. laidlawii* bilayer mixtures affects the acyl chain order and the spontaneous curvature (*19*, *20*). Analogous changes in the latter parameters can also be induced by varying phospholipid/detergent ratios in mixed amphiphile aggregates (*21*−*23*). These are properties likely to be sensed by the DGlcDAG synthase in the *A. laidlawii* membrane (*8*, *9*). Using the standard conditions (cf. above), the ratios of DOPC to different lipids with established nonbilayer potencies (*24*) were systematically varied in the mixed CHAPS aggregates. All six lipids that were used increase the spontaneous curvature and can induce a bilayer to nonbilayer transition, but only three of these stimulated the DGlcDAG synthase (Figure 5). The sugar lipids MAMGlcDAG, MADGlcDAG, and MGalDAG were tested as nonbilayer-prone, potentially stimulating lipids, but instead, they behaved as inhibitors (see above). With the cholestenone steroid, the activity increased 3-fold at a concentration of 11.5 mol %. This is in agreement with results from the crude enzyme/vesicular system where an almost 3-fold increase in activity was obtained (*8*). For the nonlamellar-forming lipid DOPE, an activity maximum appeared at 4.5 mol % DOPE, with slightly lower stimulatory power at higher concentrations. With the 1,3-dioleoylglycerol (1,3-DOG), the increase in activity was 5-fold at 11.5 mol % (Figure 5). In a crude enzyme/vesicular system, a substantial increase in the rate of DGlcDAG synthesis was achieved with 1,3-DOG present (*8*, *9*). Under conditions with saturated lipid chains in vivo, where MGlcDAG forms an L_α phase (*25*), 1,2-DAG is accumulated in the *A. laidlawii* membrane (*4*). Since 1,3-DOG has effects on phase equilibria and curvature of bilayers similar to those of 1,2-DOG (*26*), the observations here strongly indicate that the DGlcDAG synthase senses these packing properties in the mixed aggregates.

Hence, these results with the purified enzyme corroborate and extend previous findings gained from crude preparations regarding the substantial stimulation of the DGlcDAG synthase by nonbilayer lipids. However, two substrate-like, nonbilayer-prone endogenous glucolipids act as effective inhibitors at physiological concentrations of the latter.

Importance of Potassium and Phosphate. The selective stimulation of the DGlcDAG synthase by the membrane phospholipid PG was obvious (cf. above). Various organic and soluble phosphates also occur in the cytoplasm of all cells [in mycoplasmas at concentrations of 10−40 mM (*27*)]. Any electrostatic interaction between the membrane-bound synthase and a phosphate-containing molecule is likely to be quenched by other ions. The dominating ion in *A. laidlawii* is K^+ (100−200 mM; *10*). The purified DGlcDAG synthase was strongly affected by potassium phosphate (Figure 6A). The increase in activity was 8-fold at physiological phosphate concentrations, and ATP stimulated the activity even more than the phosphate. For the MGlcDAG synthase, the activity only increased 1.4-fold at 50 mM phosphate, and at higher concentrations, the activity decreased (data not shown). This stimulation of the DGlcDAG synthase was probably not given by the K^+ part of the supplement since KCl decreased the activity 1.6-fold between 100 and 300 mM (i.e., physi-

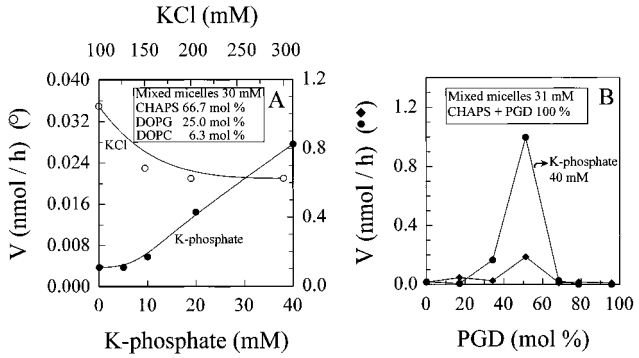

FIGURE 6: Effects of potassium and phosphate on the DGlcDAG synthase activity. Enzyme activity was analyzed as a function of (A) the KH_2PO_4/K_2HPO_4 and KCl, and (B) PGD detergent concentrations. A gradient of KCl was used in the last step of the purification (DLAC) of the enzyme.

ological concentration) (Figure 6A). Such concentrations of K^+ will substantially screen the negative charge of the activator PG present. For the MGlcDAG synthase, the KCl had a similar inhibitory effect (data not shown). These results may suggest that the glucolipid enzymes of *A. laidlawii* are dependent on, or connected to, the metabolic status of the cell. In a crude enzyme/vesicular system, ATP and other phosphorus molecules had a stimulatory effect on the DGlcDAG synthesis while MGlcDAG synthesis was inhibited (*28*). These lipid syntheses are also affected by the energetic status of the cell in vivo (*10*, *11*).

Is the phospholipid PG essential for DGlcDAG synthase activity? The detergent dodecylphosphoglycerol (PGD) has a phosphoglycerol headgroup, no glycerol backbone, and only one hydrophobic chain. PGD could restore the MGlcDAG synthase activity and an active conformation that is resistant to proteolytic digestion (*29*). Without PG and phosphate, only a small DGlcDAG synthase activity appeared at 50 mol % PGD, but with 40 mM potassium phosphate, the activity was raised 5-fold at the same PGD concentration (Figure 6B). This indicates that the access of the phosphate to the enzyme is very important for the activation; i.e., a phosphoglycerol head close to the interphase is not sufficient, whereas further out like in PG, activation is efficient. Furthermore, the enzyme can be turned on by soluble phosphate even if it is not in a mixed phospholipid/detergent micelle, and where the detergent PGD gives smaller and more highly positively curved aggregates than for the lipid/CHAPS micellar aggregates.

Efficient Regulation by Two Glucosyltransferases Reconstituted Together. Inclusion of the foreign, nonbilayer-prone molecule cholestenone strongly affects the phase equilibria and also causes compensatory responses in the membrane lipid composition in *A. laidlawii*, i.e., a decrease in the MGlcDAG fraction and a corresponding increase in the DGlcDAG fraction, both in vivo and in vitro (*8*, *30*). This is in accordance with the maintenance of the required packing properties. Is this the concerted effect of the two glucosyltransferases making these lipids?

Both the MGlcDAG and DGlcDAG synthases were reconstituted together with different cholestenone content in the mixed micelles. When the two enzymes were present, an increase in the extent of DGlcDAG synthesis and a decrease in the extent of MGlcDAG synthesis appeared

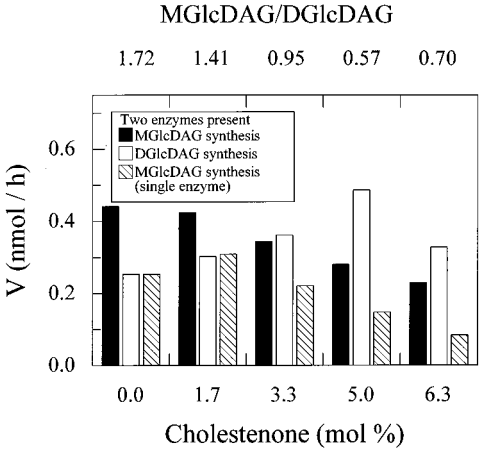

FIGURE 7: Influence of cholestenone on the amounts of MGlcDAG and DGlcDAG from two glucosyl transferases. The MGlcDAG and DGlcDAG synthases were reconstituted together in mixed micelles (30 mM) with cholest-4-en-3-one (66.6 mol % CHAPS, 25 mol % DOPG, 0 to 6.3 mol % cholestenone, and 6.3 to 0 mol % DOPC). Substrates 1,2-DOG (1 mol %), MGlcDAG (1 mol %), and UDP-Glc were present in all samples. The upper *X*-axis shows the ratio of the amount of MGlcDAG synthesized to the amount of DGlcDAG synthesized.

(Figure 7), and the MGlcDAG/DGlcDAG ratio decreased like it did in vivo. In experiments with the single MGlcDAG synthase, the activity still decreased with increasing cholestenone concentrations (Figure 7). Comparison between the single MGlcDAG synthase activity and the activity when DGlcDAG synthase was also present showed a higher activity for the latter in the presence of the MGlcDAG synthase (Figure 7). This may be a kinetic effect (the product MGlcDAG is also the substrate for the next enzyme), or the DGlcDAG synthase stimulates the MGlcDAG synthase by a direct interaction.

Hence, these results are in good agreement with the in vivo studies, and it can be concluded that these responses to the packing properties brought about by cholestenone are maintained by the pure MGlcDAG and DGlcDAG synthases.

DISCUSSION

Properties of the Purified DGlcDAG Synthase. The chromatographic techniques yielded an enrichment factor of approximately 5000 on the basis of specific activity (Table 1). This figure is probably almost twice as high given the copurification of ribosomal protein S4 (cf. Figure 2) and thus very similar to the enrichment factor for the MGlcDAG synthase (*7*), and several *Escherichia coli* lipid enzymes (*31*, *32*). The recently determined structure of protein S4 revealed a positively charged domain (*33*). Here, S4 could be selectively degraded by proteinase K under conditions preserving the high activity of the DGlcDAG synthase (data not shown); S4 is thus most likely not involved in the DGlcDAG synthesis but only copurified because of its properties.

The inclusion of polar lipids in CHAPS micelles will increase aggregate size substantially, as is the case for several closely related detergents (*16*, *34*). Here the CHAPS/lipid ratio was maintained under conditions yielding mixed micelles, and away from ratios preserving extended lipid bilayers (cf. Results and ref *15*). The amounts of DGlcDAG

synthase that were used corresponded to a concentration of ~25 nM. At a total amphiphile (CHAPS and lipid) concentration of ~30 mM, most mixed micelles will contain no enzyme even if we assume an aggregate size of several hundred molecules (*34*). Since the rate of DGlcDAG synthesis was constant with time for periods much longer than normal assay times (45 vs 5 min), an effective exchange of MGlcDAG substrate lipids between the mixed aggregates must take place. At 1 mol % MGlcDAG substrate, usually 0.01−0.075 mol % new DGlcDAG product was synthesized. With an aggregate size of, for instance, 100 molecules, 0.3 mM mixed aggregates, and 25 nM enzyme, this corresponds to 3−22.5 μM DGlcDAG, i.e., approximately 100−750 DGlcDAGs per enzyme; this is far above the value of 1 MGlcDAG (1 mol %) per aggregate. The turnover number (k_{cat}) for the DGlcDAG synthase was calculated to ~1.3 s^{-1} (at best), which yields about 780 DGlcDAGs per assay period and enzyme protein, and this is in good agreement with the numbers presented above.

Most important, several features such as dependence on PG as an activator lipid and the strong impact of bilayer curvature packing stress, previously observed in crude enzyme systems and anticipated from in vivo studies, are recorded here for the purified DGlcDAG synthase enzyme protein. Hence, the potential impact of other components is not needed to explain the in vivo responses from *A. laidlawii* (*35*). Furthermore, lipid packing responses in *A. laidlawii* may not need regulation at the gene level since the magnitudes of the responses in activity by the purified enzyme (several- to many-fold) are large enough to cope with in vivo conditions, as calculated previously (*8*).

Dependence on Activator Lipids. The dependence on an activator lipid, as shown for crude enzyme in vesicular systems (*8*, *9*, *12*), was valid also for the pure enzyme in mixed micelles. This feature could not be anticipated from in vivo and biophysical studies (*1*, *36*). Lipids with anionic headgroups (DPG and DOPS) were generally better activators than similar lipids with neutral headgroups (DOPC and DGlcDAG) for the purified DGlcDAG synthase (Figure 3). DOPS and the anionic DPG could to some extent replace DOPG as an activator in the crude systems (*12*). DOPG mixtures with small fractions of DPG or DOPS were even better here (Figure 3). Still, none of the lipids tested here could fully substitute for DOPG. Hence, the purified DGlcDAG synthase is very selective with respect to the headgroup of the lipid activator. This selectivity may indicate that the lipid activator headgroup interacts with cationic amino acid residues on the enzyme, or perhaps with an enzyme-bound divalent metal cation cofactor (e.g., Mg^{2+}) (*37*). Withdrawal of Mg^{2+} and the presence of a chelator will abolish enzyme activity (data not shown). The accessibility, or exposure of the PG head-interacting site(s), is important. Soluble phosphate stimulated the enzyme both in the bilayer-like mixed micelles (Figure 6A) and in more highly curved, detergent-only micelles (Figure 6B), in the latter case even in the absence of PG but with a PG headgroup analogue present. Hence, the actual curvature is not essential since the enzyme can be activated in different types of aggregates. The additive effects of increasing PG amounts (ref *8* and Figure 3), and increasing phosphate amounts (Figure 6B), strongly support a cooperative activation mechanism.

Enzyme Kinetics and Curvature Stress. The experimental data for the DGlcDAG synthase for the MGlcDAG lipid substrate could not be fitted to the Michaelis−Menten equation. However, a good fit with the Hill equation was obtained, showing typical cooperative kinetics. The apparent values of V_{max} and K_m at different concentrations of the activator lipid DOPG revealed that K_m was not altered (Figure 4B) whereas V_{max} was indeed changed. Similar features were identified in a crude enzyme/vesicular system (*8*), suggesting that the concentration of the activator does not affect the interaction between the enzyme and MGlcDAG substrate, but that the turnover of the reaction is modified. No synthase activity occurred unless PG, or a PG-like molecule (PGD) and soluble phosphate, was present, showing that the activator is limiting for the activity. The physiological amounts of PG determined in *A. laidlawii* (*4*) never reach the amounts yielding maximum activities with the purified enzyme here. They are more at levels coinciding with the inflection part of the sigmoidal activation curve. The DGlcDAG synthase activity was essentially constant at MGlcDAG substrate concentrations of >3 mol %, which is far below physiological concentrations. This strongly suggests that the activity of the enzyme in vivo is governed by the presence or actual amounts of the PG activator.

Lipids and amphiphiles forming or inducing nonbilayer aggregates of the reversed type usually have a fairly small (and uncharged) polar headgroup, and more bulky hydrophobic chains. In a bilayer-like environment, such molecules create a "curling" tendency for the two monolayers, toward the spontaneous curvature, sometimes referred to as a curvature packing stress (*38*). Several nonbilayer-prone molecules enhanced the rate of turnover of MGlcDAG into DGlcDAG (Figures 5 and 7). A similar influence of such packing properties on the DGlcDAG synthase was also observed in a crude vesicular system (*8*). In the latter system, the demand for the activator PG was decreased by the nonbilayer additives. Since the enzyme can be turned on or stimulated in different types of aggregates (cf. above), it is probably not the actual curvature stress that directly modulates the enzyme. More likely, (i) the access of activator PG or soluble phosphates, or (ii) the exposure of several phosphate-binding pockets (cf. Hill cooperativity) on the enzyme, is enhanced or modified by the nonbilayer curvature stimulants.

Metabolic Regulation Control Point. The three different, but nonbilayer-prone lipids 1,3-DOG, DOPE, and cholest-4-en-3-one all stimulated the DGlcDAG synthase several-fold at fairly low molar fractions (Figure 5). Such an increase in rates is sufficient to cause the metabolic increase in the amount of DGlcDAG during a 6 h period following the exposure of the cells to various nonbilayer agents (*8*). Hence, the cellular compensation in curvature stress resides partly or fully in the single DGlcDAG synthase protein; an increase in enzyme amounts, caused by an increase in the extent of transcription, may enhance the compensation further. This conclusion is strengthened by the metabolic responses to the cholestene by the MGlcDAG and DGlcDAG synthases that were reconstituted together (Figure 7). Here the MGlcDAG and DGlcDAG outputs closely mimicked observations from in vivo experiments, with strong metabolic compensation in packing properties (*8*, *30*). The increase in the MGlcDAG synthesis rate brought about by the presence of the (second) DGlcDAG synthase (Figure 7) may be caused by a chan-

neling effect; i.e., the first MGlcDAG product goes directly, as substrate, to the next enzyme. This would imply a physical contact between the two enzymes. A kinetic effect, i.e., more MGlcDAG substrate present for the second enzyme, is less likely, given the small amounts of new lipids that are synthesized by the enzymes in relation to the number of micellar aggregates and amounts of lipid substrate present from the start.

Endogenous derivatives of MGlcDAG and DGlcDAG with a third acyl chain (*2*, *3*), i.e., MAMGlcDAG and MADGlcDAG, both inhibited the DGlcDAG synthase (Figure 5) despite the fact that these lipids are both very prone to forming nonbilayer structures (*39*). However, their inhibitory potencies were substantially lower than that for β-MGalDAG (Figure 5), indicating that the two former lipids are not typical substrate competitive inhibitors. MAMGlcDAG lowered V_{max}, while K_m for the DGlcDAG synthase remained the same. A possibility is that they withdraw the MGlcDAG substrate from interacting with the enzyme, or prevent access of the activator PG. Both these nonbilayer glucolipids are probably part of a fine-tuning of the bilayer packing properties, since they are predominantly synthesized under conditions when MGlcDAG turns more bilayer-prone due to bilayer acyl chain properties (*4*, *5*). An inhibitory effect of these lipids on the DGlcDAG synthase, as shown here, will probably tilt the metabolic balance in favor of more nonbilayer-prone lipids being made (cf. the introductory section).

Hence, the purified DGlcDAG synthase maintains several regulatory features that were anticipated from the in vivo metabolism and physicochemical properties of the *A. laidlawii* membrane lipids. Similar packing conditions for lipid bilayer molecules are also crucial in other, unrelated bacteria, as indicated recently for *E. coli* (*40*, *41*), *Clostridium butyricum* (*42*), and *Bacillus megaterium* (*43*). These species have polar lipids different from and lipid hydrocarbon chains partially different from those of *A. laidlawii*, and regulation occurs mainly at the acyl chain level; for *C. butyricum* and *B. megaterium*, regulation also occurs to some extent by polar headgroup changes. In none of these bacterial systems have the sites for metabolic regulation been analyzed with purified enzymes (*32*).

From the observations here with the purified DGlcDAG synthase from *A. laidlawii* membranes, it can be concluded that the activity of the enzyme is dependent on (i) the type and concentration of a specific activator lipid, (ii) endogenous inhibitor lipids, and (iii) the bilayer curvature stress. These effects are in accordance with the global metabolic regulation in vivo. An integration of this mechanism with the metabolic state of the cell is indicated from the stimulation of the enzyme by soluble phosphates.

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