Lipid Dependence and Basic Kinetics of the Purified 1,2-Diacylglycerol 3-Glucosyltransferase from Membranes of Acholeplasma laidlawii

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UDP-glucose: 1,2-diacylglycerol 3-glucosyltransferase (EC 2.4.1.157), catalyzes the transfer of glucose from UDP-glucose to diacylglycerol (DAG) to yield monoglucoylacylglycerol (MGlcDAG) and UDP. MGlcDAG is the first glucolipid along the glucolipid pathway, and a major (nonbilayer-prone) lipid in the single membrane of Acholeplasma laidlawii. MGlcDAG is further glucosylated to give the major diglucoacylglycerol (DGlcDAG). The bilayer fractions of these lipids are crucial for the metabolic maintenance of phase equilibria close to a potential bilayer-nonbilayer transition and a nearly constant spontaneous curvature. The glucolipid synthetases are also balanced against the phosphatidylglycerol pathway, competing for the common minor precursor phosphatidic acid, to retain a constant lipid surface charge density. The 1,2-diacylglycerol 3-glucosyltransferase was purified to homogeneity from detergent-solubilized A. laidlawii cells by three column chromatography methods (enrichment ~9 000 X), and identified as a minor 40-kDa protein by using SDS-polyacrylamide gel electrophoresis. In CHAPS detergent, mixed micelles, a cooperative dependence on anionic lipids for activity was confirmed. Dependence of the enzyme on UDP-glucose followed Michaelis-Menten kinetics while UDP-glucose followed Michaelis-Menten kinetics while activity was confirmed. Dependence of the enzyme on UDP-glucose followed Michaelis-Menten kinetics while activity was confirmed.

In A. laidlawii, these lipid syntheses are regulated in response to external or internal factors influencing the physical properties of the lipid bilayer to give a strongly varying but adapted lipid composition (4). The purpose is to maintain (i) phase equilibria close to a potential bilayer-nonbilayer transition (5), (ii) a nearly constant radius of spontaneous curvature (6), and (iii) a constant (anionic) surface charge density (7) for the membrane lipid bilayer mixture. Similar packing properties are also maintained in other bacteria by regulation especially at the acyl chain level, like Escherichia coli (8, 9), Closstridium butyricum (10), and Bacillus megaterium (11), the latter two also by some extent of polar head group changes.

The regulation of the packing properties is mainly accomplished by altering the relative proportions of MGlcDAG and DGlcDAG, forming essentially nonlamellar and lamellar phases (5, 12–14). In vivo, parameters giving more nonlamellar properties to the bilayer, like higher growth temperature and more of longer and unsaturated acyl chains, result in an increase of the DGlcDAG fraction and a decrease of the MGlcDAG.

The gluelipid monoglucosylacylglycerol (MGlcDAG),1 one of the major lipids in the cytoplasmic membrane of the cell wall-less bacterium Acholeplasma laidlawii, is synthesized by glucosylation of diacylglycerol (DAG), i.e. DAG + UDP-GLC → MGlcDAG + UDP (1, 2). This is catalyzed by the membrane-bound enzyme purified and basically characterized in this work, 1,2-diacylglycerol 3-glucosyltransferase (EC 2.4.1.157) (MGlcDAG synthase).

MGlcDAG is the first glucolipid in the glucolipid pathway, and is further glucosylated by another enzyme to give diglucoacylglycerol (DGlcDAG) (1–3), the precursor for the syntheses of two (usually minor) phosphoglycerolipids. Acylated variants of the two glucolipids are also made under some conditions. The hydrophobic substrate DAG for 1,2-diacylglycerol 3-glucosyltransferase is synthesized from phosphatidic acid (PA) by the enzyme phosphatidic acid phosphatase.2 PA is also the precursor for the separate and competing pathway leading to the major (anionic) phosphatidylphosphatidylglycerol (PG).

CDP-DAG → PGP → PG

PA

DAG → MGlcDAG → DGlcDAG → phosphoglycerolipids

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1 The abbreviations used are: MGlcDAG, 1,2-diacyl-3-O-(α-D-glucopyranosyl)-sn-glycerol (monoglucosylacylglycerol); DAG, 1,2-diacylglycerol; DGlcDAG, 1,2-diacyl-3-O-(α-D-glucopyranosyl)-1→2-O-α-D-glucopyranosyl)-sn-glycerol; DO, dioloyl; DP, dipalmitoyl; DPG, sn-1,2-diacylglycerol; Glc, glucose; MGlcDAG-synthase, 1,2-diacylglycerol 3-glucosyltransferase; CDP-DAG, cytidinediphosphate-DAG; FA, phosphatic acid; PC, phosphatidylethanolamine; PG, phosphatidyglycerol; PGP, phosphatidylglycerol 3-phosphate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)l-1-propanesulfonate; IEC, ion exchange chromatography; HAC, hydroxyapatite chromatography; TLC, thin-layer chromatography; BSA, bovine serum albumin.

2 S. Berg, and A. Wieslander, manuscript in preparation.
DAG fraction (14), as do the presence of various foreign, non-bilayer-promoting molecules (15). This follows the rules for the packing and phase equilibria of amphiphiles (16, 17). In vitro, the activity of MGlcDAG synthase is critically dependent upon a lipid environment where a substantial fraction of anionic amphiphiles is present. The best activator seems to be 1,2-dioleoyl-phosphatidylglycerol (DOPG), allowing the enzymatic reaction and stimulating it in a cooperative fashion at increasing mole fractions (18, 19). This dependence of the enzyme is most likely involved in keeping the rate balance between the PG and glucolipid pathways in vivo, maintaining a constant surface charge density of the lipid membrane. The lamellar/nonlamellar balance is more likely regulated by the consecutive synthesis of DGlcDAG since the activity of this step is strongly influenced by the presence of molecules increasing the bilayer chain order or the spontaneous curvature, e.g., like certain sterols, in a manner that correlates with the regulation in vivo (19, 20).

A simplification of the crude systems used, and a purification of the proteins involved, would give valuable information about how a lipid-synthesizing enzyme is regulated by its amphiphilic environment. What properties of the environment, which are strongly altered by the nonlamellar-prone enzyme product, are sensed, and what are the regulatory responses of the enzyme? Are they in agreement with the regulation model observed in vivo and with the details accomplished from coarse experiments in vitro?

We report here the purification and identification of the MGlcDAG synthesizing enzyme 1,2-diacylglycerol 3-glucosyltransferase from A. laidlawii membranes. Confirmation of the regulatory properties of the enzyme, indicated from crude proteins in a vesicular system (18, 19), and a characterization of its dependence on the DAG and UDP-Glc substrates in a pure micellar system, add knowledge to the regulation of lipid bilayer properties.

**MATERIALS AND METHODS**

**Strain and Growth Conditions**—A. laidlawii strain A-EF22 was cultivated in an oleic acid (C18:1)-supplemented medium and harvested, and the protein content of cells and membranes was determined, as described by Dahlqvist et al. (19).

**Lipids Used in the Experiments**—Synthetic rac-1,2-dioleoyl-diacylglycerol (1,2-DOG) was purchased from Larodan (Sweden). Synthetic sn-1,2-dioleoyl-diacylglycerol (sn-1,2-DOG), sn-1,2-dipalmitoyl-diacylglycerol (sn-1,2-DPG), and bovine brain sn-1-phosphatidycholine (sn-1-PG) were purchased from Sigma (USA). DOPG, 1,2-dioleoyl-phosphatidylcholine (DOPC), 1,2-dioleoyl-phosphatidylethanolamine (DOPE), and sphingosine in 100 mM HEPES, pH 8, 20 mM MgCl2, and 0–30 mM salt were prepared as the assay micellar solutions (see below). Lipid mixtures were purified to homogeneity in CHAPS buffer (cf. above) by extensive vortexing, overnight incubation at 4°C, and bath sonication for 5 min. This caused a significant isomerization of DAG between its different chain stereoisomers, as revealed by thin-layer chromatography (TLC) analyses. The lipids were extracted from the micelle preparations into an equal volume of 1,1,1-trichloroethane and applied on Silica Gel 60 (Merck) TLC plates, which were developed in chloroform/acetone, 24:1 (v/v). Stock solutions of the diacylglycerols were used as references. Especially, 1,3-DAG was substantially isomerized into the 1,2-DAG, indicating that the 1,2-DAG was approximately 50% of the activity with sn-1,2-DAG, indicating that the sn-1,2-DAG does not act as a substrate or as an inhibitor.

In the standard assay, 5 μl of enzymatic solution (50 ng of protein) was added to 85 μl of micellar solution. The mixtures were kept for 25 min on ice and preincubated for 10 min at 25°C. Reactions were started by addition of 10 μl of UDP-[14C]glucose (37–74 GBq/mol) to give a concentration of 1 mM in a volume of 100 μl in 100 mM HEPES, pH 8, 20 mM MgCl2, and 20 mM CHAPS buffer. Standard lipid concentration was 10 mM and DAG substrate was 0.4 M NaCl, 25 mM CHAPS, and 5 mM DAG. Activity from chromatography columns was analyzed in 50-μl samples mixed with 40 μl of micellar solution to give final concentrations of 1 mM DOG, 9 mM DOPG, 110 mM Tris maleate, pH 8, 20 mM MgCl2, and 20 mM CHAPS. Micellar solution preparation and assay were the same as described above. After 5 min of incubation (or 30 min for chromatography fractions) at 25°C, reactions were stopped with 375 μl of methanol.
Lipid Dependence of Purified MGlcDAG Synthase

Enzyme Purification—Starting the enzyme purification by total solubilization of intact cells in 30 mM CHAPS by the method optimized by Karlsson et al. (18) (see "Materials and Methods"), instead of prepared membranes, substantially improved the final results and gain in activity. The stability of the enzyme was dependent on a high buffer concentration of glyc-erol (20% v/v) (cf. Ref. 18) and a low temperature (maximum of 4 °C) during the entire purification procedure. After removal of insoluble particles by centrifugation, the micelle solution was expressed as nanomoles of lipid synthesized per hour, as calculated from the amount of radioactive glucose incorporated into MGlcDAG.

RESULTS

Enzyme Purification—Starting the enzyme purification by total solubilization of intact cells in 30 mM CHAPS by the method optimized by Karlsson et al. (18) (see "Materials and Methods"), instead of prepared membranes, substantially improved the final results and gain in activity. The stability of the enzyme was dependent on a high buffer concentration of glycerol (20% v/v) (cf. Ref. 18) and a low temperature (maximum of 4 °C) during the entire purification procedure. After removal of insoluble particles by centrifugation, the micelle solution was loaded directly on an IEC column (SP-Sepharose).

The MGlcDAG synthase did bind (at pH 8 and 0.4 mM NaCl) and was essentially separated from the major peak of retained proteins by elution with a concentration gradient of NaCl (0.4–1.0 M) (Fig. 1A), giving a 190-fold enrichment. The total yield of MGlcDAG synthesis activity eluted was 47% of the activity loaded, but only 27% of the activity persisted in fractions usable for further purification (Table I). The 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 for *A. laidlawii* lipid extracts was washed out of the column before the enzyme.

By gel filtration, the enzyme activity was eluted from a Sephacryl S-100 column in two separate peaks, one before the major protein fractions and the other with them (Fig. 1B). The relative quantity of the two activity peaks varied substantially between runs, but the protein elution profiles were almost identical. This indicates an irregular behavior of the MGlcDAG synthase in this chromatography system, but there were no differences between the enzymes in the two peaks causing them to behave differently in the following hydroxycapatite chromatography procedure. However, only for enzyme from the first gel filtration peak was the specific activity improved, and these fractions, therefore, constituted the target for further purification. The activity-enrichment factor was 12, and the yield was 32% (Table I), but since the desalting and the change in buffer after the IEC gave a total yield of around 200% (for all fractions), it is likely that the actual enrichment of the enzyme by the gel filtration was lower.

The final step in the purification procedure was HAC. The best fractions from the gel filtration were pooled and directly loaded on the hydroxycapatite column. The enzyme bound firmly to the ceramic hydroxycapatite at 100 mM KH2PO4/K2HPO4, pH 8, and not until the concentration of KH2PO4/K2HPO4 was raised to ~340 mM in a gradient was the enzyme eluted from the column in a sharp, specific peak (Fig. 1C). The enrichment factor of 4.2 is probably correct since the total yield was near 100%. The achieved yield was 76% of the activity loaded on the column.

SDS-polyacrylamide gel electrophoresis of proteins in the eluted fractions revealed the occurrence of a protein of ~40 kDa, the amounts of which correlated with the elution profile of the MGlcDAG-synthesis activity (as in Fig. 1C). The gel photo in Fig. 2 shows the single protein band from a high-activity HAC fraction. The N-terminal amino acid sequence of this protein was verified from two preparations (data not shown). An enrichment factor of ~9,000 is of similar magnitude as for several purified eukaryotic and *E. coli* lipid-synthesizing enzymes, (e.g. Refs. 23 and 24). These conditions strongly indicate that the 40-kDa protein is the 1,2-diacylglycerol 3-glucosyltransferase and that it was purified to near homogeneity.

Stability and Dependence on Mg2+—The purified MGlcDAG synthase was thermo-unstable. During storage in HAC buffer, the activity half-life of the enzyme at 4 °C was around 50 h, and at 20 °C, it was only 1.8 h. At −20 and −50 °C, the enzyme was stable for at least 48 days. Since chelating and reducing agents had only small effects on the stability of the impure enzyme, they were not tested with the purified. It has previously been shown that the MGlcDAG-synthesizing (crude) enzyme is dependent on the presence of ~20 mM Mg2+ (1). A strong dependence of Mg2+ was revealed for activity of the purified enzyme, with a maximum between 15 and 28 mM MgCl2 added (data not shown). It is not possible to conclude if Mg2+ is a cofactor bound to the enzyme or if it acts by altering the charge distribution on the enzyme or the surrounding lipid surface.

Properties of Micellar Environment—It has been reported that CHAPS is poor in solubilizing (hydrophobic) lipids with small polar headgroups like DAG, requiring the presence of large headgroup lipids (preferentially anionic) in order to obtain homogenous, mixed micelles (25). The different matrix and activator lipids were investigated for their ability to solubilize the DAG substrate in mixed CHAPS micelles. DOG (0.12 mM), applied as dry films on the tube wall, was hydrated as de-
Lipid Dependence of Purified MGlcDAG Synthase

Cell solubilization, column chromatography, and assays for enzyme activity and protein concentration were performed as described under “Materials and Methods.”

| Purification step | Protein \( \mu g \) | Total activity \( \text{nmol} / \text{h} \) | Specific activity \( \text{nmol} / \text{mg} / \text{h} \) | Yield in step | Total yield | Enrichment in step | Total enrichment |
|-------------------|---------------------|-----------------|-----------------|--------------|-------------|------------------|-----------------|
| Cell solubilization | 420,000            | 32,000          | 79              |              |             |                  |                 |
| Ion exchange chromatography | 590             | 8,700           | 15,000          | 27           | 27          | 190              | 190             |
| Gel filtration | 16                 | 2,800           | 170,000         | 32           | 8.6         | 12               | 2,200           |
| Hydroxypatite chromatography | 2.9            | 2,100           | 720,000         | 76           | 6.5         | 4.2              | 9,100           |

*Figures for protein amounts and enzyme activity at each step are the amounts used in the next step.

*Figures are the sum of four separate and identical rounds of gel filtration chromatography.

**TABLE I**

**Fig. 3. Solubilization of diacylglycerol in CHAPS in the presence of different lipids.** Mixed micellar solutions were prepared as described under “Materials and Methods,” and solubilization was measured as the decrease in absorbance at 350 nm. Lipids in the mixtures (0.12 mM DOG and 1.5 mM other lipids) were: DOG only ( ), DOG and DOPG ( ), DOG and DGlcDAG ( ), DOG and DOPC ( ), and DOG and sphingosine ( ). At very low CHAPS concentrations, no stable reading of \( A_{350} \) could be obtained due to aggregation of the amphiphiles. The inset shows varying lipid concentrations in 20 mM CHAPS, with DOG and DOPG in molar ratios of 0.025 ( ) and 0.081 ( ), respectively.

**Fig. 2. SDS-gel electrophoresis of purified proteins.** Selected samples from the purification procedures stained with Coomassie Brilliant Blue: lane A, commercial reference mixture, molecular mass in kDa; lane B, solubilized whole-cell extract (3.3 \( \mu g \) of protein); lane C, high activity IEC fraction (8.7 \( \mu g \) of protein) (cf. Fig. 1A); lane D, high activity HAC fraction (cf. Fig. 1C); and lane E, like lane D, but visualized by sensitive silver staining. Gels were run (in the Bio-Rad Mini-PROTEAN II), processed, and stained as described in Nystro¨m et al. (21).

Enzyme Activity Depends on Amphiphile Concentration and Fraction of DOPG—The effect of varying DOPG/CHAPS ratio was analyzed at different total amphiphile concentrations. A larger total DOPG fraction was needed for maximal activity at higher total amphiphile concentrations than at lower ones (Fig. 4), indicating that it was the DOPG fraction in the micelles that was important for activity rather than the bulk concentration. The critical micellar concentration of CHAPS is varying with different lipid compositions, going as high as 13.7 mM (25). If an estimated CHAPS water-phase monomer concentration of 5 mM is subtracted before calculating the estimated DOPG/CHAPS ratio (in the micelles) on the x-axis (Fig. 4), the two peaks will essentially coincide. Hence, the two curves in Fig. 4 both show the same dependence of the surface concentration of DOPG in the micelles. The decrease in activity at DOPG concentrations higher than the optima (Fig. 4) might be due to a transition from one kind of micellar structure to another (perhaps bilayer-like), caused by the high lipid to detergent ratio (see Ref. 26).

A DOPG/CHAPS ratio of 0.46 was set as standard in order to minimize the risk of exceeding the optimal fraction of DOPG at 30 mM total amphiphile. This amphiphile concentration should be safely higher than the CHAPS monomer concentration.

**Activation by Anionic Lipids**—Using the standard conditions (Figs. 3 and 4), the proportions of DOPG to other polar lipids in the CHAPS micelles were systematically varied. With the zwitterionic phospholipid DOPC as the other matrix lipid, the fraction of DOPG had to be above 0.3 before any activity was detectable, and with DOPG fractions higher than 0.6, the activity increased in a sigmoidal manner (Fig. 5). The endogenous, uncharged glucolipid DGlcDAG as matrix lipid yielded essentially the same results as for DOPC (Fig. 5).
Lipid Dependence of Purified MGlcDAG Synthase

The anionic phospholipid DOPS was able to activate the enzyme to the same extent as DOPG (Fig. 5), indicating that the anionic charge is more important than the specific polar head structure. However, when DOPG and DOPS were mixed in ratios between 0.8 and 0.9, an activity maximum appeared, which implies that the overall structure of the anionic charge distribution is influencing enzyme activity. It has been shown that the activation by anionic lipids can be substantially countered by the cationic lipid sphingosine as a matrix lipid (18). This was the case also for the pure enzyme in the micellar system, but the effect was quantitatively smaller. With sphingosine as the matrix, the activation did not start until the DOPG fraction was above 0.6, and the sigmoidal appearance of activity maximum at 0.7 (Fig. 5). All in all, these result with the pure enzyme corroborates previous findings gained from crude preparations regarding the activation of the MGlcDAG synthase by an anionic lipid environment.

Kinetic Characterization of the MGlcDAG Synthase—Activity of the enzyme correlated with the UDP-Glc substrate concentration in a hyperbolic manner when UDP-Glc was altered from 0 to 3.75 mM, with a sharp raise up to 0.8 mM (Fig. 6A). The data could be fitted to the Michaelis-Menten equation with high accuracy, and the parameters \( V_{\text{max}} \) and \( K_m \) could be calculated from the fitted curve (Table II). Hence, the enzyme follows Michaelis-Menten kinetics in its dependence on the soluble substrate UDP-Glc. The calculated \( V_{\text{max}} \) is only valid for the concentration of DOG (0.8 mol %) used here, whereas the \( K_m \) remained unaltered at varying DOG concentrations. To get the absolute \( V_{\text{max}} \), one has to either saturate the enzyme with DOG or repeat the experiment at various DOG concentrations and calculate the absolute \( V_{\text{max}} \) by extrapolation. The former method is restricted by the solubility limit of DOG (cf. Fig. 7), and the latter method is only applicable if kinetics of both substrates can be described by known equations. Variation of experimental diacylglycerol concentrations was complicated since several parameters were simultaneously altered. To keep the total amphiphile concentration constant, the activator/matrix lipid or the CHAPS concentration had to be lowered, both of which have substantial influence on the activity (Fig. 3 and 4) and alter the micellar structure. However, DOG concentrations between 0.25 and 5 mol % only shift the total amphiphile concentration from 29.3 to 30.8 mM, which has a

![Graph](image1.png)

**Fig. 4.** Variation of 1,2-diacylglycerol 3-glucosyltransferase activity with amphiphile and DOPG concentration. Purified enzyme was assayed as described under “Materials and Methods” with 15 \( (\bullet) \) or 30 mol % \( (\circ) \) total amphiphile concentration where DOPG was varied at the expense of the CHAPS fraction. The DOG fraction was kept constant at 0.025 (mol/mol amphiphile).

![Graph](image2.png)

**Fig. 5.** Dependence of the 1,2-diacylglycerol 3-glucosyltransferase on anionic lipids. Enzyme activity was measured in mixed micelles with 20 mM (67 mol %) CHAPS, 0.75 mM (2.5 mol %) DOG, plus 9.3 mM (31 mol %) matrix lipid DOPC (●), DGlC-DAG (□), sphingosine (■), or DOPS (■), respectively, which were stepwise replaced by DOPG. Curves are normalized relative to their common point at a DOPG fraction of 1 (31 mol%).

![Graph](image3.png)

**Fig. 6.** Variation of 1,2-diacylglycerol 3-glucosyltransferase activity with the concentration of its two substrates. Enzyme activity was analyzed as a function of the UDP-Glc (A) and DOG concentrations (B) in the presence of 31 (●), 25 (△), and 20 mol % (○) of DOPG; in the latter two, DOPG was supplemented with 6 and 11 mol % DOPC, respectively. The amphiphile concentration was 30 mM with 9.3 mM DOPC/DOPE, plus 0.24 mM (0.8 mol %) DOG (●), and 1 mM UDP-Glc (B). The curves in A were calculated from the Michaelis-Menten equation fitted to the data points; parameters \( V_{\text{max}} \) and \( K_m \) (UDP-Glc) were calculated (Table II). Curves in B were calculated from the Hill-equation fitted to the data points. Since the data points are only covering a very small range of the calculated Hill curve, no estimation of kinetic parameters was given.

| Concentration of DOG | \( V_{\text{max}} \)  | \( K_m \) |
|----------------------|---------------------|---------|
| mol %                | nmol/h              | mM     |
| 31                   | 3.18 ± 0.09         | 0.37 ± 0.04 |
| 25                   | 2.96 ± 0.09         | 0.44 ± 0.07 |
| 20                   | 0.55 ± 0.03         | 0.39 ± 0.06 |

* Apparent values determined at 0.8 mol% of DAG and hence only valid at this concentration.

![Graph](image4.png)
Lipid Dependence of Purified MGlcDAG Synthase

Variation of the DOG concentration between 0 and 5 mol % revealed a sigmoidal correlation for the enzyme activity rather than a hyperbolic one (Fig. 6B). The data could be fitted to the Hill equation, but the parameters $V_{\text{max}}$ and $K_m$ could not be determined since the range of experimental data only covered a limited part of the calculated curve. However, it was obvious that DOG had an activating effect on the MGlcDAG synthase that could not be explained by the small increase in total amphiphile concentration. In vivo, DOG fractions are very low in membranes with unsaturated acyl chains (<1 mol %) (14).

The substrate dependence of the enzyme basically followed the same pattern at three DOPG concentrations; this was evident for both substrates (Fig. 6, A and B). Calculated values of $V_{\text{max}}$ and $K_m$ for UDP-Glc (Table II) revealed that the catalytic rate of the enzyme was altered by the variation in DOPG concentration, but that $K_m$ remained essentially the same. From this, it can be assumed that DOPG does not influence the MGlcDAG synthase activity by altering the interaction between the enzyme and UDP-Glc, but instead is affecting the turnover number of the enzyme reaction.

Hence, the MGlcDAG synthase is activated, in a potentially cooperative fashion, by its lipid substrate, DAG, and it follows Michaelis-Menten kinetics with respect to its other soluble substrate, UDP-glucose. The activator DOPG does not affect the interaction of the enzyme with UDP-glucose but increases the turnover of the enzymatic catalysis in a cooperative manner.

The Type of Acyl Chains in Diacylglycerol Substrate Are Important—Enzyme activities with a racemic mixture of DOG were at all concentrations approximately half of the activity gained with a pure sn-1,2-dioleoylglycerol, which indicates that the sn-2,3-diacylglycerol is neither a substrate nor an inhibitor of the enzyme. Analyses with sn-1,3-DAG on thin layer chromatography showed that a substantial fraction of the 1,3 variant was isomerized into the other chain forms during the micelle preparation; this is well known to occur in aqueous systems (27). Consequently, the racemic-1,2-DAG used in most of the experiments here gave less variable assay results than the sn-1,2-diacylglycerol since it probably was in equilibrium with the sn-2,3-isomer from the start.

sn-1,2-Dipalmitoylglycerol (DPG) as substrate revealed twice as high enzyme activity as sn-1,2-dioleoylglycerol (DOG) at all concentrations up to 2.5 mol % concentration (Fig. 7). At this point, the MGlcDAG synthase activity decreased at increasing DPG concentrations. The breaking point was most likely due to segregation of DPG into separate domains or aggregates when the solubility limit of DPG in 20 mM CHAPS and 9.3 mM DOPG was exceeded. Such a breaking point also existed for DOG, but occurred first at 10 mol % (data not shown). Thus, for the activation of the enzyme by DAG, the upper accessible (experimental) concentration of the substrate has a limit that is far below the concentrations that should represent $K_m$ or give $V_{\text{max}}$ according to the calculated curves in Fig. 6B. The activity at the limiting concentration may be regarded as the absolute $V_{\text{max}}$ in this particular experimental system, and the highest specific activity recorded for the MGlcDAG synthase in this system was 2 mmol/mg/h. Such a rate is, as estimated from the total yield of purified enzyme activity (Table I), in many-fold excess of what should be required for supplying fast-growing cells with enough MGlcDAG for the glucolipid pathway (given from Nyström et al. (21)).

These results are in agreement with results from the crude enzyme vesicular system where DPG was a better substrate at low concentration and DOG was the better one at high concentrations (19). Hence, the acyl chain preference for the DAG species is valid also for the purified MGlcDAG synthase.

### DISCUSSION

In order to maintain a functional membrane, A. laidlawii adapts the lipid composition to shifting environmental and nutritional conditions. A constant surface charge density, similar phase equilibria, and a constant spontaneous curvature are thereby kept by the concerted actions of the lipid-synthesizing enzymes. However, the properties of the lipid bilayer that are sensed by such enzymes is less well known (e.g. Ref. 23). In A. laidlawii membranes, the major nonlamellar lipid MGlcDAG is synthesized by transfer of a glucose moiety from UDP-glucose to DAG, catalyzed by the enzyme 1,2-diacylglycerol 3-glucosyltransferase. MGlcDAG is also the first glucolipid in the glucolipid pathway. The variations in activity of this enzyme upon different properties of the amphiphilic environment is, therefore, of particular interest and has been studied earlier in crude cell preparations (2, 18, 19).

**Enzyme Purification**—The purification method used here consists of three column chromatography steps in a CHAPS micellar environment. Basically, it relies on the first cationic exchange chromatography, leading to a 190-fold increase (relative to solubilized cells) in the specific activity (Fig. 4A and Table I). The identification of the enzyme in SDS-polyacrylamide gels as a minor 40-kDa protein was possible only after a several thousand-fold enrichment in activity and is mainly based on the correlation between protein gel-band intensity and enzyme activity in different fractions and by a single protein in the final high activity fractions. This is supported by a resistance against digestion by proteinase K for both the enzyme activity and this protein band. Resistance is dependent on the presence of anionic lipids like DOPG and PS, as is the enzyme activity, corroborating the identification.

**The CHAPS Micellar System**—It was revealed that the activity of the purified MGlcDAG synthase required a certain fraction of DOPG in the CHAPS micelles (Fig. 4) as does in the crude preparations (18). There seems to be a correlation between the packing properties of the lipids and their solubility in CHAPS. The hydrophobic, nonbilayer substrate lipid DAG, known to increase packing constraints and acyl chain order in lipid bilayers, had a poor solubility in CHAPS (Figs. 3 and 7) (see also Maréchal et al. (28)). However, if DAG were mixed with large mole fractions of lipids with larger polar headgroups, it could more easily be solubilized by CHAPS (Fig. 3). This was evident for all of the lipids used as activator or matrix lipids in this study. The slight differences between them in solubilization of DAG at low CHAPS concentrations (Fig. 3) did

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3 L. Li, O. P. Karlsson, and Å. Wieslander, manuscript in preparation.
not affect the DAG solubility at assay conditions, as revealed by the similarity between the different curves in Fig. 5.  

Lipid Dependence of the Enzyme—Many membrane-associated enzymes are dependent upon activator lipids, often anionic ones, for example the protein kinase C (29–32) and the phospholipase C (33, 34). The MgGlcDAG synthase seems to have a dependence on a critical fraction of anionic lipids (18, 19). The lipid best suited to fulfill this requirement was DOPG, the major endogenous anionic phospholipid in membranes of A. laidlawii (18).

None of the uncharged (DGlcDAG), neutral-charged (DOPC), or positive-charged (sphingosine) lipids could replace DOPG as activator for the purified MgGlcDAG synthase (Fig. 5). On the other hand, the anionic phospholipid PS could fully substitute for DOPG; DOPG mixtures with small fractions of PS were even better. The surface (or $\zeta$) potential of the lipid aggregates must be efficiently quenched by the Mg$^{2+}$ present, cf. McLaughlin (35). Together this indicates that it is the anionic-charged groups of the activator lipids that hold the activating property, as proposed earlier (18, 19).

These results essentially confirm the earlier findings from both crude vesicular and mixed micellar preparations and strengthens the hypothesis that the MgGlcDAG synthase demands a critical fraction of anionic lipid charges in its amphiphilic environment in order to be active. The sigmoidal appearance of the activation curves was also evident, indicating that the enzyme is activated by the same mechanism in both crude and pure systems.

Interpretation of the Kinetics and Comparison with Similar Systems—The variation in activity of the MgGlcDAG synthase with altering bulk DAG concentration at fixed CHAPS and DOPG concentrations followed a sigmoidal path (Fig. 6B), indicating an activating ability of DAG. This is according to the dual phospholipid model of Hendrickson and Dennis (36), a strong indication that the reaction involves more than one type of interaction with DAG where one is a binding step to the micellar surface. However, the MgGlcDAG synthase is behaving as a protein anchored to the amphiphilic matrix by hydrophobic interactions (3, 5), but DAG may, as well, promote interactions (i) between (an external) catalytic domain and the lipid surface, or (ii) between enzyme monomer subunits. Both alternatives are also compatible with a cooperative mechanism. The Hill equation could be more accurately fitted to the data than could the equation suggested by Hendrickson and Dennis (36), but since the data only represented a very small range of the calculated curves, none of the equations can be ruled out and no parameters could be calculated with certainty. DAG might also function as a noncooperative activator, as it does for the E. coli diacylglycerol kinase in the absence of activating phospholipids (37). Although the enzyme product MgGlcDAG is a potent non-bilayer-prone lipid, the activity data from the various lipid supplements and the DPG and DOG substrates argues against any (major) influence of the spontaneous curvature on activity (or activation) of the MgGlcDAG synthase at physiologival DOPG concentrations. This is in accordance with crude-enzyme results (18–20).

The dependence of the MgGlcDAG synthase on its soluble substrate UDP-glucose was more easily interpreted since the data could be fitted to the Michaelis-Menten equation, and the concentration of UDP-Glc could be varied to give data points in all essential parts of the curve (Fig. 6A). A comparison of the apparent values of $V_{\text{max}}$ and $K_m$ at different concentrations of the activator DOPG (Table II) revealed that the $K_m$ was not altered. $V_{\text{max}}$, on the other hand, was changed in accordance with the activation by DOPG, as shown in Fig. 5. The enzyme is thus following Michaelis-Menten kinetics with UDP-Glc, and the concentration of DOPG does not affect the interaction between the enzyme and UDP-Glc, but the turnover of the enzyme is substantially affected. The apparent $V_{\text{max}}$ was increased almost 6-fold by altering the DOPG fraction from 20 to 31 mol %, i.e. at physiological concentrations. An 11-mol % variation in DOPG at these conditions will only have minor effects on the charge density and the resulting surface ($\zeta$) potential, as deduced from several other studies (7, 35, 38, 39). This strongly indicates that the activation is not linearly dependent on the lipid surface charge concentration.

Importance for the Regulation Model of A. laidlawii—This study supports the regulation model for A. laidlawii lipid synthesis (3, 19) where the MgGlcDAG synthesis is enhanced by the negative surface charge brought by DOPG and two (usually minor) phospholipids. Since MgGlcDAG is the first glucolipid along the glucolipid metabolic pathway, the regulation will act to maintain a balance to the phospholipid (i.e. PG) pathway, competing for the same PA precursor. The model is in accordance with the ability of the purified MgGlcDAG synthase to be activated by a critical fraction of anionic lipids in the micellar system (Fig. 5). Interestingly, a dependence on anionic lipids for the in vivo synthesis of the major nonlamellar-prone lipid in E. coli has recently been suggested (40).

The higher efficiency of DPG than of DOG as a substrate is also in accordance with both the regulation model and the crude vesicular system (19). The amounts of MgGlcDAG increases strongly with short acyl chains in vivo (14, 41), and this must be governed by a substrate preference of the enzyme for such chains since the MgGlcDAG synthesis is only weakly regulated by properties affecting chain order and curvature (see above and Ref. 19).

From these observations on the purified MgGlcDAG synthase, it can be concluded that the regulatory properties of the enzyme step are held by one single protein and are probably not part of a complex multiprotein regulatory mechanism. The interaction of this enzyme with its amphiphilic environment can now be investigated.

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Lipid Dependence of Purified MGlcDAG Synthase

936

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