Activating Amphiphiles Cause a Conformational Change of the 1,2-Diacylglycerol 3-Glucosyltransferase from *Acholeplasma laidlawii* Membranes According to Proteolytic Digestion*

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1,2-Diacylglycerol 3-glucosyltransferase synthesizes the major nonbilayer-prone lipid monoglucoosyldiacylglycerol (MGlcDAG) in the membrane of *Acholeplasma laidlawii*, which is important for the spontaneous curvature, and is a regulatory site for the lipid surface charge density. A potential connection between activity and a conformational change of this enzyme, governed by essential lipid activators, was studied with purified MGlcDAG synthase in different lipid aggregates.

Critical fractions of anionic phospholipids, 1,2-dioleoyl-phosphatidylycerol (DOPG) and 1,2-dioleoyl-phosphatidylinerine (DOPS) were essential for the restoration of enzyme activity, while the zwitterionic 1,2-dioleoyl-phosphatidylcholine (DOPC) and the anionic di-glucosyldiacylglycerol (DGlcDAG) were not. Proteolytic resistance had a very good correlation with the enzyme activity in various lipid-CHAPS mixed micelles. Anionic lipids DOPG and DOPS could protect the exposed MGlcDAG synthase from digestion, whereas DOPC and DGlcDAG could not. Similar features were observed in liposome bilayers. Likewise, the detergent dodecylphosphoglycerol (PGD), with a phosphatidylycerol-like headgroup, could also stimulate the MGlcDAG synthase activity efficiently with a concomitant protection toward proteolytic digestion. Neither proteolytic resistance nor restored enzyme activity was observed using soluble glycerol 3-phosphate. It is concluded that in addition to critical amounts, both the negatively charged headgroup and hydrophobic chains of the activator amphiphiles, but not a certain aggregate curvature, seem necessary for a proper conformation and the resulting active state of the MGlcDAG synthase.

The roles of native lipids are more and more evident in the function of membrane proteins, especially the influence of such lipids on protein conformation and folding state (e.g. 1, 2). In *Acholeplasma laidlawii* membranes, the major phospholipids and glucolipids are extensively regulated under a variety of conditions to maintain certain properties like (i) a constant surface charge density (3), (ii) a nearly constant spontaneous curvature (4), and (iii) phase equilibria close to a potential membrane bilayer to non-bilayer transition (5). These properties seem to be sensed and regulated by the different lipid-synthesizing enzymes involved. The pathways of the syntheses in *vivo* of the major lipids (in bold) are as follows.

\[ \text{CDP-DAG} \xrightarrow{\text{PGP}} \text{PG} \]
\[ \text{PA} \xrightarrow{\text{DAG}} \text{MGlcDAG} \xrightarrow{\text{DGlcDAG}} \text{2 Phosphoglycerolipids} \]

**Scheme 1.**

MGlcDAG, a major nonbilayer-forming lipid synthesized from DAS, is the first glucolipid in the glucolipid pathway; it is catalyzed by 1,2-diacylglycerol 3-glucosyltransferase (MGlcDAG synthase). The major, bilayer-forming glucolipid DGlcDAG is consecutively synthesized from MGlcDAG (6–8). Derivatives of these two glucolipids, with an extra, third acyl chain may also be synthesized under certain conditions *in vivo* (9). The MGlcDAG synthase is dependent upon a lipid environment where a critical fraction of anionic amphiphiles, such as PG, must be present (10). This dependence of the enzyme seems 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 bilayer. The lamellar/nonlamellar balance and curvature are more likely regulated by the synthesis of DGlcDAG because this step is strongly affected by nonbilayer-forming additives *in vitro* (11), thus increasing the turnover of the nonbilayer-prone MGlcDAG into the bilayer-forming DGlcDAG.

Anionic lipids were essential for the recovery of MGlcDAG synthase activity in both membrane vesicles and for pure enzyme in micelles (7, 10, 11). This followed a similar lipid dependence for certain soluble proteins activated by membrane surface association, e.g. protein kinase C (12, 13) and phosphocholesterol cytidylyltransferase (14). However, the MGlcDAG synthase is firmly anchored in the membrane and only solubilized efficiently by detergents and certain chaotrophic salts. Hence, activation must involve changes of either the membrane-bound enzyme or its local environment, like altered protein conformation or aggregation state, or lipid domain formation, respectively.

Limited proteolysis by proteases is a useful method for the study of protein conformation (15–17) since such enzymes preferentially cleave at exposed regions between structural domains. Here we apply this to investigate the digestion resistance of the MGlcDAG synthase in different amphiphile

*The abbreviations used are: MGlcDAG-synthase, 1,2-diacylglycerol 3-glucosyltransferase; DAG, 1,2-diacylglycerol; DGlcDAG, 1,2-diacyl-3-O-[a-D-glucopyranosyl[1–2]O-a-D-glucopyranosyl]-sn-glycerol; DO, dioleoyl; DOG, sn-1,2-dioleoylglycerol; MGlcDAG, 1,2-diacyl-3-O-[a-D-glucopyranosyl]-sn-glycerol; PA, phosphatidic acid; PC, phosphatidylylycerol; PG, phosphatidylethanolamine; PDD, dodecylphosphoglycerol; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; FM, fluorescein-5-maleimide; BM, benzophenone-4-maleimide; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

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1 O. P. Karlsson, unpublished data.
mixtures. A very good correlation was observed between the extent of digestion resistance and enzyme activity for the MGlcDAG synthase. This indicates that between the active and inactive states in differently composed micelles, there is most likely a conformational change of this enzyme. The detergent dodecylphosphoglycerol (PGD), which has a headgroup similar to PG (18), could also efficiently enhance the enzyme activity with a concomitant proteolytic resistance. Hence, an amphiphilic environment with a critical fraction of negatively charged headgroups induces a catalytic, active conformation of the MGlcDAG synthase.

EXPERIMENTAL PROCEDURES

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.* (11).

Lipids and Other Materials—MGlcDAG and DGlcDAG were prepared from *A. laidlawii* grown in the presence of oleic acid, which gives polar lipids with more than 90% (mol/mol) C18:1 acyl chains (11). Synthetic rac-1,2-dioleoylglycerol (1,2-DOG) was purchased from Larodan (Sweden). DOPG, DOPC, and DOPS were purchased from Avanti Polar Lipids, and PGD was from Alexis (Switzerland). Immobilized proteinase K was purchased from Merek (Germany), and trypsin and chymotrypsin were from Boehringer Mannheim (Germany). Fluorescein-5-maleimide (FM) and benzophenone-4-maleimide (BM) were from Molecular Probes.

Purification of MGlcDAG Synthase—Homogeneous MGlcDAG synthase was purified from detergent-solubilized *A. laidlawii* cells by three column chromatography methods, including ion exchange, gel filtration, and hydroxyapatite chromatography, according to the procedure by Karlsson *et al.* (10). "Detergent-free" enzyme was accomplished using the same procedure with only the last method modified. Here, the buffers used for eluting the hydroxyapatite chromatography column were changed to be devoid of the detergent CHAPS. No analysis of CHAPS remains was done. However, if no high-affinity binding of CHAPS to the enzyme occurs, the elution procedure is expected to remove all detergent from the column.

Preparation of Liposomes—Lipid mixtures with desired composition were first dried under a stream of nitrogen, and then put in vacuum overnight to remove trace amounts of organic solvents. The lipids were then hydrated in buffer containing 20 mM HEPES and 20 mM MgCl₂, pH 8. The mixtures were vortexed vigorously at room temperature until they became emulsified. These multilamellar vesicles were made and used the same day.

Assay for MGlcDAG Synthase Activity—In micelles, the assays were performed as described before (10). For the assays in liposomes, MGlcDAG synthase was added to prepared liposomes and kept on ice for 30 min, followed by preincubation at 28 °C for 3 min. Enzyme reactions were started by addition of UDP-[^C]glucose (37–74 GBq/mol). After 10 min of incubation at 28 °C, the reaction was stopped by addition of methanol/chloroform 2:1 (v/v), and lipids were extracted and subsequently separated by thin layer chromatography (8, 19). Synthesized MGlcDAG was quantified by electronic autoradiography (Packard Instant Imager™).

Limited Proteolytic Digestion of MGlcDAG Synthase—Lipids were mixed and dried under a stream of N₂ and then under vacuum for 2 h. The lipid mixtures were totally solubilized in 20 mM Tris-maleate, 20 mM MgCl₂, 20 mM CHAPS, pH 8, plus/minus 9 mM DOPG for 30 min in ice. Immobilized proteinase K (3–0.1 unit) was added to the mixed micelles or prepared liposomes. The mixtures were gently vortexed at 28 °C for 10 min. The proteolysis was terminated by removing the immobilized proteinase K using centrifugation at 2000 × g, and supernatants were then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Likewise, 0.02 μg of trypsin or 0.1 μg of chymotrypsin was used to digest MGlcDAG synthase in lipid micelles at 28 °C for 10 min, respectively. Here, the reactions were stopped by addition of 1 mM phenylmethylsulfonyl fluoride.

Denaturation of the Enzyme by Urea—The enzyme was incubated with a different concentration of urea in 110 mM Tris-maleate, 20 mM MgCl₂, 20 mM CHAPS, pH 8, plus/minus 9 mM DOPG for 30 min on ice. Proteinase K is resistant to fairly high urea concentration. Then the samples were used for the activity assays and proteolytic digestion in the presence of DOPG as in the methods described above.

Cysteine Labeling of the Enzyme—Purified MGlcDAG synthase was treated with 10 mM DTT at 30 °C for 10 min. Excess DTT was then removed by using dialysis overnight against 110 mM Tris-maleate, 20% glycerol, 20 mM CHAPS, 0.1 mM DTT, pH 8. A lipid micellar solution containing 10 mM lipids, 20 mM CHAPS, 20 mM MgCl₂, 110 mM Tris-maleate, pH 8, was mixed with 0.1 μg of MGlcDAG synthase for 30 min on ice. Fluorescein-5-maleimide or benzophenone-4-maleimide was added to 2.5 mM, and samples were incubated at 30 °C for 2 h, respectively. The reactions were stopped by adding 2% (v/v) 2-mercaptoethanol (20, 21). Proteins were extracted from the micellar solution by partitioning in organic solvent (22) and analyzed by SDS-PAGE.

Protein Gel Electrophoresis—SDS-PAGE was performed using 15% acrylamide in the resolving gel for proteolytically digested samples (23). Proteins were visualized by silver staining. Quantification of protein bands in stained gels was carried out using an ImagerMaster™ (Pharmacia LKB). The relative intensity of the stained enzyme bands was presented as the relative proteolytic resistance.

RESULTS AND DISCUSSION

Dependence of Activity and Proteolytic Resistance on Anionic Lipids in Micelles—The MGlcDAG synthase has a strong dependence on a critical fraction of anionic lipids both in micelles and in bilayers. Uncharged, neutral charge, or positively charged lipids could not function as activators (7, 10). The relative activity and proteolytic digestion resistance of the MGlcDAG synthase in different lipid-CHAPS micelles are shown in Fig. 1. The resistance against proteinase K digestion in the different mixed micelles was visualized by SDS-PAGE (Fig. 1B). With no lipids present, the MGlcDAG synthase could be totally digested. However, the enzyme revealed highly restored activity in DOPG- and DOPS-containing micelles, but no restored activity could be observed in DOPC- and DGlcDAG-containing micelles nor in CHAPS only. The lipids without negative charge, like DOPC and DGlcDAG, could not protect MGlcDAG synthase from proteolysis, whereas DOPG and DOPS could efficiently protect the enzyme from digestion. The same protective ability of DOPG could also be observed by using the proteases trypsin and chymotrypsin (data not shown).

The activity and proteolytic resistance of the MGlcDAG synthase varied in a correlated manner when the fraction of the activator DOPG in the lipid matrix (DGlcDAG or DOPC) was...
changed (Fig. 2). The enzyme activity was enhanced when the DOPG ratio in total lipids was increased while the enzyme became more resistant to the digestion by proteinase K. However, it was found that by extending incubation times the enzyme could also be digested at the most protective conditions, i.e., the longer the proteolytic time the more digested was the enzyme, even in the presence of DOPG.

Potential, large changes in orientation or exposure of the micelle-associated enzyme was monitored by the labeling of cysteine residues, present in local hydrophobic or hydrophilic environments, with two specific agents. Small and medium mass proteins reveal easily detectable changes of migration distance in SDS-PAGE when labeled with the large and bulky agents FM (hydrophilic) and BM (hydrophobic), respectively (21). However, no changes in labeling with these two agents, binding to hydrophilic and hydrophobic regions, respectively, were observed (data not shown) as a function of different lipid composition (cf. Fig. 1). Hence, the overall membrane topology of the enzyme is most likely not changed by the lipid environment, but an orientational change of an exterior (hydrophilic) domain is possible. Furthermore, cross-linking studies in mixed micelles strongly indicate that the active enzyme is a monomer.3

The results above show that there is correspondence between the activity and proteolytic digestion resistance for the MGlcDAG synthase. When the enzyme showed high activity, it also had high digestion resistance in mixed lipid micelles. Some other proteins have also been found to have similar features. Anionic phospholipids could recover activity and protect the FAD-dependent malate dehydrogenase from Mycobacterium smegmatis, while zwitterionic phospholipids could not. At the same time, little secondary structure change could be detected (24). Phospholipase A₂ is known to associate much more favorably with a negatively charged membrane surface compared with a zwitterionic one, with a conformational change upon activation (25).

Dependence of Enzyme Stability on Certain Lipids—The thermostability of MGlcDAG synthase in different micelles also varied according to the lipid environment. Fig. 3 shows the correlation between the thermostability and digestion resistance of the enzyme. With prolonged incubation at 28 °C, the enzyme activity became lower both in DOPG and DOPC micelles, but the half-life differed. The half-time at 28 °C in DOPG-CHAPS micelles was more than 2 h, while in CHAPS and DOPC-CHAPS micelles it became less than 0.5 h. This suggests that the activator DOPG could stabilize the enzyme efficiently against thermo-deactivation. Similar features were also shown by the proteolysis experiments. Enzyme with low activity, due to longer incubation times both in the presence and in the absence of DOPG, also revealed a low digestion resistance.

Does the negatively charged DOPG inhibit the activity of proteinase K? The denaturant urea was used to study whether the susceptibility of active or denatured MGlcDAG synthase to proteinase K was affected by DOPG since urea does not inhibit the activity of proteinase K (see “Experimental Procedures”). MGlcDAG synthase denatured by 3 mM urea for 30 min on ice had no activity at 28 °C, and it could be digested totally by proteinase K even if DOPG was added after the urea incubation while the enzyme without urea showed high activity and digestion resistance at the same conditions. In 1 mM urea, a low activity and proteolytic resistance were preserved (data not shown). It is also evident from Fig. 3 (no urea) that the MGlcDAG synthase became more easily digested by subsequently added proteinase K by prolonged time at 28 °C in the presence of DOPG. These results suggest that the negatively charged molecule PG itself does not interfere with proteinase K. When the enzyme was held at 28 °C for more than 2 h, DOPG could neither restore the activity nor protect the enzyme from digestion. In this case, both the deactivation and the conformational changes of the enzyme were irreversible. In contrast, the conformational changes by different amphiphile composition were reversible. This reversibility was valid both for activity and proteolytic resistance, which then must result from a conformational change of the enzyme.

Stimulation by PGD Detergent—In lipid-CHAPS mixed micelles, the aggregate curvature might be influenced by the ratio between the bilayer-forming polar lipids and the micellar CHAPS. Hence, the enzyme stimulation may result from the negatively charged PG molecules but be influenced by the curvature of mixed-micelles.

Potential enzyme activity in more highly curved aggregates were analyzed by using the micelle-forming detergent PGD, which has a negatively charged headgroup similar to PG but only one hydrophobic chain. Around 30% of PGD in total amphiphiles could restore the MGlcDAG activity (Fig. 4), and the highest restored activity was about the same as with DOPG as the activator. Proteolytic resistance was found to correlate with the activity also in PGD-CHAPS micelles (Fig. 4), similar to the PG-CHAPS micelles. However, an excess amount of activator PGD could neither stimulate the activity nor protect enzyme from digestion, which was also observed with excess amounts of DOPG. The enzyme activity became higher with the PG con-

3 L. Li, O. P. Karlsson, and Å. Wieslander, unpublished observation.
tent, but a content of over 33% (mol %) decreased the enzyme activity (10). Proteolytic resistance in PG-CHAPS micelles showed the same pattern of variation.

Glycerol 3-phosphate, a hydrophilic molecule with a negatively charged phosphate group, and an isomer of the PG head, could not restore the enzyme activity when used as a potential activator up to 2 mM. This suggests that a hydrophobic interaction with the activator, or a surface position of the negatively charged amphiphile headgroup, is important for the protein conformation and activity. It can be concluded that it is anionic amphiphiles like PG, PS, or PGG, but not the aggregate curvature or the negatively charged headgroup only, that are essential for the restoration of a proper conformation and enzyme activity of the MGlcDAG synthase.

Dependence of Activity and Proteolysis Resistance on Anionic Lipids in Bilayers—Can the stiff CHAPS molecule confer any conformational features to the enzyme? The purified MGlcDAG synthase, prepared free from CHAPS, was used to study how PG affects the enzyme in liposome bilayers (without CHAPS). Fig. 5 shows that the activity was enhanced by increasing the PG content in liposomes. Activity could be restored, and the enzyme showed increasing digestion resistance to protease K when PG content was raised over 20% (mol/mol). The catalytic domain of the enzyme might bind to the bilayer at activating fractions of DOPG initially by an electrostatistic interaction and subsequently by more firm hydrophobic interactions. The latter can be of a slower rate compared with the former. It can be concluded that an electrostatistic interaction seems necessary for the restoration of the enzyme activity and the protective, catalytic conformation of the enzyme also in bilayer membranes.

A strong dependence of enzyme activity on the Mg²⁺ concentration, with a maximum at ~20 mM, has been recorded (10). However, from proteolytic experiments it was shown that PG in CHAPS micelles could protect the enzyme from digestion also in the absence of Mg²⁺ (data not shown). It is thus likely that Mg²⁺ does not contribute to the tight conformation or binding of the enzyme in PG-CHAPS micelles. Another possibility is that Mg²⁺ alters the charge distribution on the enzyme or in the surrounding lipid surface or that the enzyme has a specific Mg²⁺ site. Neither of the substrates, 5 mol % 1,2-DOG and 1 mM UDP-glucose, seemed to have any evident influence on the conformation of the enzyme, as detected by proteolysis protection in micelles (data not shown).

The results presented in this report show that activating amphiphiles could protect the MGlcDAG synthase from proteolytic digestion. However, longer digestion times in proteolytic experiments will result in nearly total digestion of the enzyme even in DOPG activator (data not shown). Similar behaviors were also found for the proteolytic susceptibility of ribonuclease A (26) and annexins IV and VI (27). The interaction between MGlcDAG synthase and the anionic activator amphiphiles could be of electrostatic origin and make part of the enzyme bind rather tightly to the membrane or micelle surface, and/or cause a more compact enzyme conformation. Both alternatives can make the enzyme less accessible to proteases.

The MGlcDAG synthase is not considered to be very hydrophobic since it can be dissolved without detergent and can be released from A. lairdiiucii membrane by the chaotropic salt KSCN. However, as reported here, the amphiphilic property of the activator is necessary for the restoration of catalysis activity and the active state or conformation. Hence, there might be a hydrophobic anchor domain in the enzyme that is permanently inserted into the membrane interior, like in many eukaryotic glucosyltransferases (28). The activity of the enzyme may be governed by the association of the exterior, hydrophilic domain of the enzyme with a properly charged (anionic) membrane surface. This activation is, from the data presented here, strongly indicated to involve significant conformational changes of the enzyme.

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**Fig. 4.** The influence of PGD on activity and proteolytic resistance. The enzyme activity (●) and digestion resistance toward proteinase K (○) were measured with different PGD fractions in micelles (30 mM total amphiphiles). Curves are normalized relative to their common point at 50 mol % PGD.

**Fig. 5.** Dependence of activity and proteolytic resistance of the MGlcDAG synthase on DOPG content in bilayers. The enzyme activity (●) and digestion resistance toward proteinase K (○) were measured in liposomes. DOPG was stepwise exchanged for DOPG at 10 mM total lipids and 0.6 mM DOG substrate. The curve is normalized relative to the point where the DOPG content was 50 mol %.
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