BIOSYNTHESIS OF THE PHOSPHATIDYL DIGLUCOSYL DIGLYCERIDE OF STREPTOCOCCUS FAECALIS (ATCC 9790) FROM DIGLUCOSYL DIGLYCERIDE AND PHOSPHATIDYL GLYCEROL OR DIPHOSPHATIDYL GLYCEROL

Ronald A. Pieringer

Department of Biochemistry
Temple University School of Medicine
Philadelphia, Pennsylvania 19140

Received September 1,1972

Summary

A phosphatidyl-group from either phosphatidyl glycerol or diphosphatidyl glycerol is covalently bonded to diglucosyl diglyceride to form phosphatidyl diglucosyl diglyceride in a reaction readily catalyzed by a disrupted membrane enzyme preparation from Streptococcus faecalis.

Previous studies from our laboratory have shown particulate enzyme preparations from Streptococcus faecalis catalyze the sequential synthesis of monoglucosyl diglyceride, diglucosyl diglyceride, and phosphatidyl diglucosyl diglyceride (fig. 1) from two common precursors, UDP-glucose and diglyceride (1,2). The last reaction in the sequence, which took place readily,

diacylglycerol-(3←1'α)-glucose-(2'←1"α)-glucose
|
6'-phosphoryl-diacylglycerol

figure 1: phosphatidyl diglucosyl diglyceride

was assumed to occur by the donation of a phosphatidyl group from an unidentified, endogenous substrate to the diglucosyl diglyceride (2). Studies on the identity of the endogenous substrate are presented here and show that both phosphatidyl glycerol and diphosphatidyl glycerol can function as phosphatidate donors in the synthesis of phosphatidyl diglucosyl diglyceride.

METHODS: The conditions of growth of Streptococcus faecalis and the preparation of particulate membrane-derived enzymes for the in vitro incubations used in this study have been previously described (1,2). Diglucosyl diglyceride, labeled with ^{14}C-glucose, was made by an in vitro enzymatic reaction between UDP-

(^{14}C)-glucose and diglyceride (1). Diglucosyl diglyceride, phosphatidyl glycerol and diphosphatidyl glycerol labeled with ^{14}C exclusively in the glycerol moieties were synthesized *in vivo* by growing *S. faecalis* in the presence of (^{14}C-1,3-)-glycerol under conditions similar to those previously described (2). Treatment with glacial acetic acid specifically hydrolyzed the ^{14}C-glycerol labeled phosphatidyl glycerol to ^{14}C-diglyceride and ^{14}C-glycerol phosphate (3,4). Each of these two products contained the same amount of radioactivity demonstrating that both of the glycerols of the phosphatidyl glycerol were equally labeled. Since phosphatidyl glycerol is the precursor of diphosphatidyl glycerol, probably by a reaction between two phosphatidyl glycerols (5), it is likely that the glycerols of diphosphatidyl glycerol will also be equally labeled. The specific radioactivity of the glycerol derived from ^{14}C-phosphatidyl glycerol by alkaline and acid hydrolysis was determined to be 70 cpm per nmole as assayed by periodate oxidation (6) and a Geiger counter with an efficiency of about 25%.

The above radioactive substrates and the enzyme reaction products were purified and identified by the following chromatography and electrophoresis systems. Purification of intact lipids was carried out by (system A) silicic acid column chromatography according to Vorbeck and Marinetti (7) and (system B) on silicic acid impregnated paper (Whatman SG-81) developed with diisobutyl ketone-acetic acid-water (40:25:5 v/v) (8). Water-soluble compounds derived by deacylation of the lipids used in this study were separated readily on Schleicher and Schuell 2040-B paper in the presence of pyridine-acetic acid-water (1:10:89 v/v) subjected to 50 volts per cm for 65 min (system C). Under these conditions the distances traveled from the origin by the deacylated lipid derivatives were the following: diglucosyl glycerol (from diglucosyl diglyceride) 0 cm; glyceryl phosphoryl diglucosyl glycerol (from phosphatidyl diglucosyl diglyceride), 13.5 cm; glycerol phosphoryl glycerol (from phosphatidyl glycerol), 22.7 cm; and diglycerol phosphate glycerol (from diphosphatidyl glycerol), 31 cm. Other systems were Whatman No. 1 developed ascending with butanol-pyridine-water (6:4:3 v/v) (system D) and Whatman No. 1 developed with propanol-ammonia (Sp gr. 0.9)-water

Table I

Biosynthesis of Phosphatidyl Diglucosyl Diglyceride

Exp. No.	Tube No.	Substrates (^{14}C-Glycerol labeled)	Lipid Products PDGD	PG	DPG
			(cpm on electropherogram)		
1	1	DGD	624	0	0
1	2	PG	157	-	1255
1	3	DPG	0	0	-
1	4	DGD + PG	1093	-	1551
1	5	DGD + DPG	2163	249	-
2	1	DGD	1750	0	0
2	2	DPG	248	632	-
2	3	DGD + DPG	3655	1275	-
3	1	DGD + PG	992	-	2123
3	2	DGD + DPG	2331	905	-

Abbreviations: DGD = diglucosyl diglyceride; PG = phosphatidyl glycerol; DPG = diphosphatidyl glycerol; and PDGD = phosphatidyl diglucosyl diglyceride. Experiment 1. 16,392 cpm ^{14}C-DGD,8,680 cpm ^{14}C-DPG, and 35,226 cpm ^{14}C-PG (all labeled in the glycerol moieties and having an estimated specific activity of 70 cpm per nmole of glycerol) were added in benzene solutions to 5 mg of a lyophilized 30,000 x g particulate preparation of S. faecalis (containing 1.4 mg protein) in the combinations indicated. After evaporation of the benzene, 0.02 ml 0.1 M $MgSO_4$ and 0.2 ml 0.1M $NaPO_4$ pH 7.3 were mixed with the (^{14}C)-lipid-enzyme complex. The mixtures were incubated for 1 hr. at 37°. Controls containing no enzyme were also incubated and produced no PDGD. The reaction was stopped with 1 ml methanol, heated for 1/2 min at 100°, chilled, and diluted with 2 ml $CHCl_3$ and 1 ml H_2O. The lipids in the $CHCl_3$ phase were deacylated with mild base (9,2) and the resulting water-soluble products separated by electrophoresis (System C). The radioactivity at each separated product (detected by a strip scanner) was determined in a Geiger counter and is expressed as the sum of the counts on both sides of the paper. Experiment 2: Conditions as in Exp. 1 except the cpm of ^{14}C-DPG was increased to 43,200 and 3 mg of an older enzyme preparation containing 0.3 mg protein were used. Experiment 3: Conditions as in Exp. 1 except 8,196 cpm ^{14}C-DGD, 34,700 cpm ^{14}C-DPG, and 35,226 cpm ^{14}C-PG were used.

(6:3:1 v/v) (system E). Standard diglucosyl diglyceride and phosphatidyl diglucosyl diglyceride were prepared under conditions in which the structures have been proven (1,2). Standard phosphatidyl glycerol and diphosphatidyl glycerol were obtained from commercial sources.

The incubation and assay conditions for the enzymatic synthesis of phosphatidyl diglucosyl diglyceride are given in Table I and are similar to those used previously (2).

RESULTS: As previously shown (2) ^{14}C-diglucosyl diglyceride interacts with an endogenous substrate in the presence of a particulate enzyme preparation from S. faecalis to produce ^{14}C-phosphatidyl diglucosyl diglyceride (Tubes 1, Exp. 1 and 2, Table 1), which has the detailed structure shown in figure 1. The addition of either ^{14}C-phosphatidyl glycerol or ^{14}C-diphosphatidyl glycerol to the ^{14}C-diglucosyl diglyceride and enzyme preparation results in the stimulation of ^{14}C-phosphatidyl diglucosyl diglyceride synthesis (Table 1). This increase in synthesis is greater than the sum of the amount of ^{14}C-phosphatidyl diglucosyl diglyceride synthesized by any of ^{14}C-substrates incubated alone and indicates that diglucosyl diglyceride and phosphatidyl glycerol or diphosphatidyl glycerol may be mutually stimulatory in the formation of the phosphoglucolipid. To determine if the phosphatidyl glycerol and diphosphatidyl glycerol do interact with diglucosyl diglyceride and function by donating a phosphatidyl group the incubations of Table 1 were repeated except that the diglucosyl diglyceride substrate was labeled with ^{14}C-U-glucose rather than ^{14}C-glycerol. Under these conditions the phosphatidyl diglucosyl diglyceride product should contain both ^{14}C-glycerol (from either phospholipid substrate) and ^{14}C-glucose (from the glucolipid substrate). After deacylation and separation in system C, the phosphoglucolipid product made from ^{14}C-glucose and ^{14}C-glycerol labeled substrates was further hydrolyzed with 2N HCl for 3 hrs. at 100°. The hydrolysis mixture together with about 0.5 μmole each of carrier glucose, glucose-6-phosphate, glycerol, and glycerol phosphate were chromatographed in system D. Three ^{14}C-peaks were detected by a chromatogram scanner: one at the origin, which chromatographed with standard glucose phosphate and glycerol phosphate; a second large peak at 9 cm from the origin, which was the distance standard glucose traveled; and a third smaller peak at 14 cm, which chromatographed with standard glycerol (the standards were detected by periodate-starch (10) and $FeCl_3$-sulfosalicylic acid (11) stains). The radioactivity at the origin was eluted

with water and electrophoresed in system C. A ^{14}C-peak was detected and it ran with standard glycerol phosphate at 21.5 cm from the origin and not with glucose phosphate at 18.5 cm. The acid hydrolysis products derived from the deacylated phosphatidyl diglucosyl diglyceride were also chromatographed along with standards in System E. ^{14}C-glycerol and ^{14}C-glucose were identified by cochromatography at 18.3 cm and 14 cm from the origin, respectively. Glycerol phosphate and glucose phosphate standards at 5 cm did not separate in this system (E). However, the radioactive material in this area was identified as glycerol phosphate and not glucose phosphate in system C. Chromatography of the enzymatically synthesized phosphoglucolipid in system B showed that it ran with standard phosphatidyl diglucosyl diglyceride at 18 cm from the origin. These data indicate that phosphatidyl glycerol and diphosphatidyl glycerol can donate a phosphatidyl group to diglucosyl diglyceride to form phosphatidyl diglucosyl diglyceride.

DISCUSSION: Based on the mechanism of acid hydrolysis of a phosphodiester bond (12) and the finding of only glycerol phosphate and no glucose phosphate in the acid hydrolysis products, the phosphodiester bond of the phosphatidyl diglucosyl diglyceride synthesized from phosphatidyl glycerol or diphosphatidyl glycerol and diglucosyl diglyceride must be at carbon-6 of one of the glucose moieties. At any other position a free adjacent alcohol function would be available for the formation of a cyclic phosphate intermediate and glucose phosphate as well as glycerol phosphate should result. Carbon-6 is the location of the phosphodiester bond in the phosphoglucolipid (fig. 1) made from diglucosyl diglyceride and endogenous substrate. These preliminary data indicate a specificity in the placement of the phosphatidyl group and suggest the detailed structure of the phosphatidyl diglucosyl diglyceride made from diglucosyl diglyceride and exogenous phospholipid-substrate will prove to be the same as shown in fig. 1.

Although a phosphatidyl group for phosphatidyl diglucosyl diglyceride synthesis can be derived from either phospholipid substrate, the data of Table I do not prove that both do so directly since the crude enzyme preparation catalyses the formation of phosphatidyl glycerol from diphosphatidyl glycerol and vice versa

during the incubation. However, if diphosphatidyl glycerol is the direct donor of a phosphatidyl group then phosphatidyl glycerol should be the other product of the reaction and might be expected to increase in concentration during the synthesis of phosphatidyl diglucosyl diglyceride. The data (Exp. 1, tube 5 and 3, and Exp. 2, tube 3 and 2, Table I) appear to indicate that this is the case. If phosphatidyl glycerol were an obligatory intermediate then its concentration probably would decrease. Whether the phosphatidyl glycerol formed in this reaction is also a substrate remains to be proven. Diphosphatidyl glycerol appears to be a better substrate (by about twofold in Exp. 3, Table I) than phosphatidyl glycerol.

It should be noted that the substrates used in these studies were derived from S. faecalis. Commercially prepared phosphatidyl glycerol and diphosphatidyl glycerol isolated from other organisms did not appear to function as substrates.

Although CDP-diglyceride can not be eliminated as a possible substrate, extensive attempts by our laboratory (unpublished) to test this possibility have failed to show a substrate function for CDP-diglyceride. The formation of phosphatidyl diglucosyl diglyceride in S. faecalis appears to be similar to the synthesis of diphosphatidyl glycerol in E.coli (5) where a preformed phospholipid rather than a nucleotide intermediate is the preferred phosphatidyl-group donor.

ACKNOWLEDGEMENT: Expert technical help was provided by Miss Erlinda Cabacungan. This work supported by research grants AI-05730, 1-PO1-HD-05874, and NS-10221 from the U.S. Public Health Service and GB 24802 from the National Science Foundation. The author gratefully acknowledges the receipt of Research Career Development Award NS-12776.

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