Diglyceride Kinase Mutants of Escherichia coli: Inner Membrane Association of 1,2-Diglyceride and Its Relation to Synthesis of Membrane-Derived Oligosaccharides

CHRISTIAN R. H. RAETZ* AND KARL F. NEWMAN

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received for publication 3 October 1978

Mutants of Escherichia coli defective in diglyceride kinase contain 10 to 20 times more sn-1,2-diglyceride than normal cells. This material constitutes about 8% of the total lipid in such strains. We now report that this excess diglyceride is recovered in the particulate fraction, primarily in association with the inner, cytoplasmic membrane. The diglyceride kinase of wild-type cells was recovered in the same inner membrane fractions. The conditions employed for the preparation of the membranes did not appear to cause significant redistribution of lipids and proteins. The biochemical reactions leading to the formation of diglyceride in E. coli are not known. To determine whether diglyceride formation requires concurrent synthesis of the membrane-derived oligosaccharides (H. Schulman and E. P. Kennedy, J. Biol. Chem. 252:4250-4255, 1977), we have constructed a double mutant defective in both the kinase (dgk) and phosphogluco isomerase (pgi). When oligosaccharide synthesis was inhibited in this organism by growing the cells on amino acids as the sole carbon source, the diglyceride was no longer present in large amounts. When glucose was also added to the medium, the pgi mutation was bypassed, oligosaccharide synthesis resumed, and diglyceride again accumulated. These findings suggest that diglyceride may arise during the transfer of the sn-glycero-1-P moiety from phosphatidylglycerol (and possibly cardiolipin) to the oligosaccharides. In wild-type cells the kinase permits the cyclical reutilization of diglyceride molecules for phospholipid biosynthesis.

In Escherichia coli phosphatidic acid is generated either by the two-step acylation of sn-glycero-3-P or by the phosphorylation of sn-1,2-diglyceride (13, 19, 21, 23, 24, 30, 32). The acylation reactions represent the major de novo pathway for the biosynthesis of membrane phospholipids (13, 21), whereas the kinase appears to be a relatively minor route (23). As shown in previous studies from this laboratory, mutants defective in the kinase accumulate 10 to 20 times more diglyceride than is present in wild-type bacteria (23). In the best available mutant (RZ60, see Table 1), about 8% of the total lipid is diglyceride during exponential growth at 37°C (23). Although not temperature sensitive, RZ60 fails to divide on media of low osmolarity (23).

We now report that diglyceride accumulation in the kinase mutants occurs primarily within the cytoplasmic membrane. Like most enzymes of phospholipid synthesis (3, 21, 33), diglyceride kinase is also tightly associated with the inner membrane fraction. For resolving the inner and outer membranes, we have employed the technique of Osborn and Munson (17), which affords excellent separations for both RZ60 and an isogenic wild-type strain designated RZ600. As shown below, significant rearrangement of phospholipids and proteins does not occur under these conditions.

The biochemistry of diglyceride formation in E. coli has not been studied previously. Possible sources include a specific phosphatidic acid phosphatase (30), a phospholipase C (16, 20), and the enzyme(s) involved in the generation of the membrane-derived oligosaccharides (MDO) (9, 26, 31). The latter is a family of glucose-containing compounds present in the periplasmic space, to which sn-glycero-1-P moieties are attached via phosphodiester linkages (9, 31).

Kennedy and co-workers have presented evidence that the sn-glycero-1-P unit arises from the polyglycerophosphatides, i.e., phosphatidylglycerol or possibly cardiolipin (9, 25, 26, 31). The transfer of this moiety to MDO precursors could generate sn-1,2-diglyceride as a by-product.

The synthesis of the oligosaccharides can be blocked without inhibiting cell growth (25, 26),
for instance, when mutants defective in phosphoglucone isomerase (pgi) are grown on amino acids as the carbon source (26). In the absence of glucose, such mutants cannot make the oligosaccharides, and the turnover of the polyglycerophosphatidates is greatly reduced (26). In the present work we have constructed a double mutant defective in both phosphoglucone isomerase and diglyceride kinase. When this strain is grown on amino acids, relatively little diglyceride accumulates unless glucose is added to the medium to permit MDO synthesis. Thus, we postulate that much of the diglyceride of E. coli arises in conjunction with the synthesis of the MDO, although additional sources cannot be excluded. The presence of the kinase in wild-type organisms constitutes a diglyceride recycling system within the cytoplasmic membrane.

MATERIALS AND METHODS

Materials. [γ-32P]ATP, 32P,33P-orthophosphate, sodium [1-14C]acetate, [2-14C]glycerol, and Triton X-100 were products of New England Nuclear Corp., Boston, Mass. Phosphatidylethanolamine labeled with 14C in the fatty acid moieties was prepared from cells that were grown in the presence of [1-14C]acetate as described below and was purified on a column of silicic acid (29). sn-1,2-Diolen and beef heart cardiolipin were obtained from the Sigma Chemical Co., St. Louis, Mo.

Bacterial strains. The construction of strains RZ600 (dgk-6) and RZ6000 (dgk*) is described elsewhere (see Table 1) (23). Strain RZ600 is wild type with respect to the kinase (dgk) gene, whereas strain RZ600 is defective in the kinase, resulting in the accumulation of substantial amounts of diglyceride in the membrane (23). Strain RZ76 (Table 1) is defective both in phosphoglucone isomerase (pgi-2) and in diglyceride kinase (dgk-6). The other strains listed in Table 1 are either obtained from the E. coli genetic stock center of Yale University or are constructed as indicated by transduction procedures described previously (14, 23).

Conditions of cell growth and labeling. For the purpose of preparing isolated inner and outer membrane fractions (17), the cells were grown on a rich broth (PPBE) consisting of 10 g of proteose peptone

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Markers</th>
<th>Source (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES 430</td>
<td>HfrH thi-1 malB29 relA1</td>
<td>CGSC* (23)</td>
</tr>
<tr>
<td>RZ600</td>
<td>HfrH thi-1 mal* dgk-6 relA1, by transduction of ES 430</td>
<td></td>
</tr>
<tr>
<td>RZ6000</td>
<td>HfrH thi-1 mal* dgk* relA1, by transduction of ES 430; iso- genic with RZ60</td>
<td>(23)</td>
</tr>
<tr>
<td>DF2000</td>
<td>pgi-2 zufA2 relA1 tonA22</td>
<td>CGSC</td>
</tr>
<tr>
<td>RZ76</td>
<td>thi-1 pgi-2 dgk-6, transductant of RZ60 (DF2000 donor)</td>
<td>This work</td>
</tr>
<tr>
<td>RZ75</td>
<td>thi-1 pgi-2 dgk-6, transductant of RZ60 (DF2000 donor); iso- genic with RZ76</td>
<td>This work</td>
</tr>
</tbody>
</table>

CGSC, E. coli Genetic Stock Center, Yale University, New Haven, Conn.

Lipid extraction. Phospholipids were extracted from whole cells or from isolated membrane fractions with chloroform and methanol by the method of Ames (1, 23). Crude E. coli phosphoglycerides and 1,2-diolen were each included as carrier during these extractions at a level of 0.5 mg/ml of chloroform. Separation of neutral lipids and phospholipids was accomplished by chromatography on silicic acid columns as reported previously (23).

Enzymatic assays. The activity of NADH oxidase was determined spectrophotometrically (17). Conditions for the radiochemical detection of diglyceride kinase were those of Schneider and Kennedy (23, 24). The activity of phospholipase A, an enzyme associated with the outer membrane, was measured by the method of Nishijima et al. (15), except that Triton X-100 was included at a level of 0.1%, and the specific activity of phosphatidylethanolamine was 4,000 cpm/nmol. The conditions of Fraenkel and Levison were used to assay phosphoglucone isomerase (5, 6).

Separation of inner and outer membranes. Both mutant and wild-type strains grew well on PPBE broth (17). All methods applicable to wild-type strains were equally effective for mutants lacking the diglyceride kinase, and no modifications of the procedures reported by Osborn and Munson (17) were necessary. Disruption of the lysosome- and EDTA-treated spheroplasts (17) was achieved by three ultrasonic bursts, each lasting 15 s, with a W185F ultrasonic disrupter (Heat Systems, Inc.) equipped with a no. 419 microtip. Sonic disruption was performed while the sample was immersed in an ice-salt bath (17). One-minute intervals were allowed for cooling between each sonic oc- cillation (80 to 100 W of power output). Membrane isolation and sucrose gradient centrifugation were performed as described by Osborn and Munson (17). Each gradient was divided into about 28 fractions. The overall recoveries of phospholipids and marker en- zymes from the gradients were over 90% relative to the material applied to the gradients and varied be- between 60 and 80% relative to the sonically disrupted, cell-free extracts.

Miscellaneous methods. Protein concentration was determined by the method of Lowry et al. (11). The radioactivity of membranes and isolated lipid fractions was determined by liquid scintillation counting in 10 ml of Patterson-Green fluid (18). The com- position of the proteins in the isolated membrane fractions was assessed by gel electrophoresis in the presence of sodium dodecyl sulfate (10). The gels were compared visually after staining with Coomassie brilli- ant blue G and destaining in acetic acid.
Stable dispersions of E. coli diglyceride and E. coli phospholipids were prepared in 3-ml portions by sonic oscillation of the lipid (5 mg/ml) in a solution containing 0.25 M sucrose, 3.3 mM Tris-hydrochloride (pH 7.8), and 1.0 mM EDTA by using the W185F disrupter (see above) with the no. 419 microtip at maximal intensity. Four 30-s bursts were employed while the sample was immersed in ice water, and 1-min intervals were allowed for cooling.

RESULTS

Subcellular localization of 1,2-diglyceride in strain RZ60. Three methods of disrupting bacteria were used to assess the subcellular localization of the diglyceride that accumulates in RZ60. These were: (i) sonic oscillation, as described under experimental procedures; (ii) a single passage through a French pressure cell at 10,000 lb/in²; and (iii) osmotic lysis by the method of Osborn and Munson (17). Cell-free extracts were subsequently analyzed by differential centrifugation, and in all three cases over 95% of the diglyceride was recovered in the particulate fraction sedimenting after 60 min at 100,000 × g.

These results suggest that the neutral lipids found in RZ60 are not sequestered in the cytoplasm as fat droplets, because these would float upon ultracentrifugation. In addition, staining of mutant cells with Sudan black (12) fails to reveal the presence of cytoplasmic fat droplets.

Separation of inner and outer membranes. Membrane fragments prepared by sonic disruption were separated by isopycnic sucrose gradient centrifugation as shown in Fig. 1. Outer membranes were detected by assaying for phospholipase A, whereas inner membranes were detected by measuring NADH oxidase (Figs. 1A and C). Excellent resolution of these markers was obtained for both the mutant and the wild-type strains. Total chloroform-soluble lipid was determined radiochemically, because the cells were labeled with [1-14C]acetate for six to eight generations before membrane isolation. As shown in Fig. 1B and D, membranes of both RZ60 and RZ600 were separated into two major, phospholipid-containing peaks, corresponding to inner and outer fragments (cf. Fig. 1A with B and Fig. 1C with D). Recovery of phospholipids and marker enzymes was comparable in both strains, ranging between 60 and 80% relative to the cell-free extract. The specific activity of NADH oxidase was somewhat lower in all fractions obtained from the mutant (Table 2).

The distribution of 1,2-diglyceride between the inner and outer membranes differed markedly from that of the total phospholipid. In the wild type (Fig. 1B) more diglyceride was recovered in the outer membrane, although the absolute amount was relatively low and somewhat variable (factor of two) from experiment to experiment. The inner membrane of the wild type contained no more than 0.7% diglyceride. In the mutant (Fig. 1D) a striking accumulation of diglyceride occurred primarily in the cytoplasmic membrane, at a level which consistently represented between 12 and 14% of the total chloroform-soluble material. The chromatographic methods for identifying diglyceride have been published previously (23).

Distribution of diglyceride kinase. The fractions obtained in the experiment shown in Fig. 1A were assayed for diglyceride kinase activity. The kinase was recovered primarily with the inner membrane, because the kinase profile (Fig. 2) was very similar to that of the NADH oxidase (Fig. 1A). In addition, the purifications of diglyceride kinase and NADH oxidase were comparable relative to the crude extract (Table 2). Accumulation of diglyceride in RZ60 was most pronounced in the same membrane fractions that contained the highest level of kinase activity in the wild type (cf. Fig. 1D with Fig. 2).

Phospholipid and protein composition of isolated membranes. The peaks from the gradients of Fig. 1A and C were further analyzed for their phospholipid and membrane protein compositions. A slightly higher percentage of phosphatidylethanolamine was present in the outer membrane in both cases, but the mutant and wild type did not differ greatly with regard to their overall phospholipid composition (Table 3), except for the lower amount of cardiolipin in the mutant, noted previously (23). The protein composition of the isolated membranes was also nearly identical in RZ60 and RZ600, as judged by a visual comparison of stained polyacrylamide gels (data not shown).

Effect of sonic oscillation on the subcellular distribution of phospholipids and neutral lipids. Several experiments were carried out to exclude the possibility of lipid exchange between inner and outer membrane fragments during cell disruption and further handling. In the experiment of Fig. 3A, 0.3 ml of an inner membrane preparation from RZ60 labeled with [1-14C]acetate (fraction 16 of Fig. 1D) was added to a standard preparation of non-radioactive spheroplasts (17) of RZ60 just before sonic disruption. The membranes were then prepared and isolated as usual (17), except that the peaks of inner and outer membrane material were located by their turbidity (Fig. 3A). When this sucrose gradient was analyzed for its lipid radioactivity, over 90% of the 14C was reisolated with the inner membranes (Fig. 3A), consistent with the view that phospholipid exchange between membrane fragments was negligible during sonic...
DIGLYCERIDE CYCLE IN E. COLI

DIGLYCERIDE CYCLE IN E. COLI

Fig. 1. Separation of inner and outer membranes from RZ600 (dgk') and RZ60 (dgk-6). Membranes from a culture of 200 ml of cells growing on PFBE (17) in the presence of [1-14C]acetate were prepared as described in the text and were suspended in a final volume of 1.0 ml of Tris buffer (17). A sample (0.8 ml) was layered on a six-step sucrose gradient, the bottom of which contained 55% sucrose and the top of which contained 30% sucrose (17). The gradients were centrifuged for 14 to 16 h at 36,500 rpm in a Beckman SW 41 Ti swinging-bucket rotor at 4°C. Fractions of approximately 0.4 ml were collected by piercing the bottom of the tubes and allowing the contents to drain by gravity. (A) and (B) show the distribution of marker enzymes and lipids, respectively, for a gradient separation of membranes isolated from RZ600, and (C) and (D) show similar results of a gradient separation of membranes from RZ60. The accumulation of diglyceride in RZ60 does not alter the apparent buoyant density of the inner membrane. The lipid-to-protein ratios, as judged by [1-14C]acetate incorporation and Lowry assay, do not differ significantly in the membranes of RZ60 and RZ600.

oscillation. Phospholipid rearrangement was also minimal when 0.3 ml of labeled outer membranes (fraction 5, Fig. 1D) was included during sonic disruption of unlabeled cells, as shown in Fig. 3B. In both cases (Fig. 3A and B) the small amount of lipid that was transferred to the other membrane fraction was not enriched in 1,2-diglyceride.

If dispersions of E. coli diglyceride or crude phospholipids were exogenously added to unlabeled spheroplasts just before sonic disruption (final concentrations of 50 to 100 μg/ml), very little of the exogenous lipid (less than 2%) became associated with the membranes under the conditions employed (data not shown). However, over 95% of the endogenous diglyceride of RZ60 was found to be membrane bound (see above).

Short-term radiochemical labeling of diglyceride in RZ60. When cells of wild-type E. coli are exposed to sn-glycero-3-[32P]P or [2-3H]glycerol for 10 or 20 s, the intermediates of de novo phospholipid synthesis, such as phosphatidic acid and CDP-diglyceride, are labeled preferentially (4, 21, 22). In contrast, the diglyceride of RZ60 is labeled less effectively in such
short-term experiments (Table 4) than in more prolonged or in "pulse-chase" experiments (Table 4). The lag in the labeling of the diglyceride (Table 4) suggests that this substance is a relatively late metabolite and certainly not an early biosynthetic intermediate.

**Relationship of diglyceride accumulation in RZ60 to the synthesis of the MDO.** As noted above, the MDO of *E. coli* are linked as phosphodiester to sn-glycero-1-P moieties (9), which might arise from the polar headgroups of phosphatidyglycerol or possibly cardiolipin (25, 26). If the diglyceride also arises during the transfer of the sn-glycero-1-P unit to the oligosaccharides, then the inhibition of MDO synthesis should reduce the amount of diglyceride that accumulates in mutants lacking the kinase.

To test this possibility, we constructed strain RZ76, which is defective both in diglyceride kinase and in phosphoglucone isomerase. This was done by treating strain RZ60 (thi-1 pgii- dgk-6) with a P1vir lysate prepared on DF2000 (thi+ pgii-2 dgk+). The observed distribution of the kinase is similar to that of NADH oxidase (Fig. 1A), suggesting that the kinase is primarily an inner-membrane protein.

Table 2. Purification of membranes from RZ600 (dgk+) and RZ60 (dgk-6)

<table>
<thead>
<tr>
<th>Fraction (strain)</th>
<th>Sp act (U/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADH oxidase</td>
</tr>
<tr>
<td>1. RZ600 (dgk+)</td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>0.29</td>
</tr>
<tr>
<td>Washed membranes</td>
<td>0.8</td>
</tr>
<tr>
<td>Inner membranes</td>
<td>3.3</td>
</tr>
<tr>
<td>Outer membranes</td>
<td>0.03</td>
</tr>
<tr>
<td>2. RZ60 (dgk-6)</td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>0.13</td>
</tr>
<tr>
<td>Washed membranes</td>
<td>0.4</td>
</tr>
<tr>
<td>Inner membranes</td>
<td>1.3</td>
</tr>
<tr>
<td>Outer membranes</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Enzyme assays: NADH oxidase, 1 U = 1 μmol/min at 25°C, phospholipase A and diglyceride kinase, 1 U = 1 nmol/min at 37°C. 

**Table 3. Phospholipid composition of inner and outer membranes isolated from RZ600 (dgk+) and RZ60 (dgk-6)**

<table>
<thead>
<tr>
<th>Fraction (strain)</th>
<th>PE</th>
<th>PG</th>
<th>CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>RZ600 (dgk+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact cells</td>
<td>82.1</td>
<td>16.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Outer membranes</td>
<td>90.7</td>
<td>8.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Inner membranes</td>
<td>74.3</td>
<td>23.5</td>
<td>2.4</td>
</tr>
<tr>
<td>RZ60 (dgk-6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact cells</td>
<td>80</td>
<td>19.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Outer membranes</td>
<td>91.3</td>
<td>8.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Inner membranes</td>
<td>68.3</td>
<td>31</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Cells were grown on PPBE broth and labeled with [1-14C]acetate as described in the legend to Fig. 1. Values for isolated membranes represent the average of the three peak fractions (Fig. 1). The phospholipid composition of the intact cells, shown above, is in good agreement with the compositions observed for cells grown on M56 medium (23). Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin. Percentage of total refers to the sum of PE, PG, and CL.
medium supplemented with 0.4% Casamino Acids, no MDO were detected by chemical aniline

For this purpose the total MDO fraction was prepared by gel filtration as described by Schulman and Kennedy (25, 26). The relative level of diglyceride was also much lower in RZ76, as judged by long-term acetate labeling, being 2.4% instead of the 7 to 9% (Table 5) typical of single

mutants carrying only the dgk-6 lesion (23). However, when 0.2% glucose was added to an otherwise identical culture of RZ76, the level of diglyceride rose to 7% (Table 5), and MDO was again synthesized in normal amounts (0.5 to 1% of the dry weight). The neutral lipid contents of RZ600 (pgi+ dgk+) and of strains RZ60 and RZ75 (both pgi+ dgk-6) growing on 0.4% amino acids were not greatly affected by the addition of glucose to the medium (Table 5), and these strains were all capable of making the normal amount of MDO in the presence or absence of glucose.

The level of diglyceride determined by acetate labeling is an accurate reflection of the true lipid composition, because essentially the same results shown in Table 5 are obtained when total lipid and diglyceride are quantitated by chemical ester determination (23). The chemical amount of lipid per milligram of protein is nearly identical in RZ60 and RZ600 and represents 6 to 7% of the dry weight of the cell.
Table 5. Effect of the phosphoglucose isomerase (pgi 2) mutation on neutral lipid accumulation in a diglyceride kinase mutant

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>% Neutral lipid*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RZ600</td>
<td>dgk&quot; pgi&quot; thi-1</td>
<td>0.6</td>
</tr>
<tr>
<td>RZ60</td>
<td>dgk-6 pgi-1 thi-1</td>
<td>9.6</td>
</tr>
<tr>
<td>RZ75</td>
<td>dgk-6 pgi-1 thi'</td>
<td>9.7</td>
</tr>
<tr>
<td>RZ76</td>
<td>dgk-6 pgi-2 thi'</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* Neutral lipid was determined by long-term labeling of cells growing on M56 salts supplemented with 0.4% Casamino Acids, using 0.3 mM [1-14C]acetate as described previously (23). To test the effect of glucose on neutral lipid content, long-term labeling was carried out on the same medium, except that glucose was also included at a level of 0.2%. Essentially the same results shown above were obtained when neutral lipid was determined by chemical ester analysis (23, 28). Over 90% of the neutral lipid fraction is 1,2-diglyceride.

**DISCUSSION**

Like all lipids of *E. coli* (21), 1,2-diglyceride is associated exclusively with the particulate fraction and is not found in the cytoplasm or in the growth medium. This conclusion is based on the observed membrane localization, which does not depend on the method used to disrupt the cells, and on the fact that neutral lipids are not present in the cytoplasm by histochemical criteria. In contrast to the diglyceride that builds up in RZ60 (Fig. 1), exogenous diglyceride dispersions added during sonic oscillation of the cells do not associate in significant amounts with the particulate fraction (sedimenting at 150,000 x g after 4 h), and lipid rearrangement between inner and outer membranes does not occur under the conditions employed in the present work (Fig. 3). These findings support the view that the inner membrane localization of the diglyceride found in the kinase mutants is physiologically significant, although the possibility of artifacts introduced during cell breakage can never be excluded completely in studies of subcellular localization. In this regard, it should be noted that the apparent outer membrane localization of diglyceride in the wild type (Fig. 1) is less convincing, because the low level of diglyceride extracted from the wild type is variable (by about a factor of two) and seems to be somewhat higher (1 to 1.5%) after spheroplast formation than in the intact cells (0.5 to 0.8%).

The association of diglyceride kinase with the inner membrane (Fig. 2) provides further support for the significance of the inner membrane localization of the diglyceride in RZ60. Like de novo phospholipid synthesis itself (3, 21, 33), diglyceride reutilization appears to occur primarily on the inner membrane. The active site of the kinase is probably oriented inwards, because ATP is available in the cytoplasm.

There are several explanations for the striking association of diglyceride with the inner membrane of RZ60 (Fig. 1). First, the diglyceride may be more soluble in the lipids of the cytoplasmic membrane than of the outer membrane and may become associated with the inner membrane, after being formed elsewhere in the cell. This mechanism seems improbable, however, because the lipid compositions of the inner and the outer membranes are relatively similar (Table 3). Alternatively, the factor(s) responsible for the translocation of lipids from their site of synthesis on the inner membrane to the outer membrane may be selective for phospholipids and may not readily accept diglyceride molecules. An in vitro system capable of lipid translocation between inner and outer membrane fragments would be desirable for studying this problem. Whatever the true explanation may be, diglyceride seems to differ from phospholipids, which have equal access to both membranes and can move back and forth under various circumstances (7, 8, 21).

For instance, exogenous phospholipid vesicles fused with deep-rough mutants of *Salmonella typhimurium* are recovered equally in both membranes and can be metabolized by enzymes present on the inner membrane (7, 8). Furthermore, minor phospholipids, such as phosphatidylserine and cardiolipin (C. R. H. Raetz, unpublished data), that accumulate in certain mutants defective in phospholipid biosynthesis distribute themselves relatively evenly between both membranes (21).

As indicated above, the reactions which may be responsible for the formation of diglyceride in *E. coli* include phosphatidic acid phosphatase (30), phospholipase C (16, 20), and the enzyme(s) involved in the transfer of the sn-glycero-1-P moiety from polyglycerophosphatides (such as phosphatidylglycerol) to the MDO (9, 26, 31). The correlation between diglyceride accumulation and MDO synthesis shown in Table 5 strongly suggests that the latter is a major source of diglyceride in wild-type cells. However, other sources may exist, because the diglyceride content of the double mutant does not completely return to wild-type levels (Table 5), even when MDO synthesis is totally blocked. Furthermore, a small fraction of the MDO contain phosphorylketanolamine residues (9), and thus some of the diglyceride could arise from phosphorylketanolamine. In any case, the kinase presumably functions as a salvage enzyme to permit the reutilization of diglyceride molecules. This is important, because most of the ATP required for membrane phospholipid synthesis is ex-
Fig. 5. A diglyceride cycle in E. coli. Diglyceride molecules may be generated during the transfer of the sn-glycero-1-P moiety from phosphatidylglycerol to precursors of the MDO. When MDO synthesis is blocked by means of the pgi mutation, the accumulation of diglyceride in the kinase mutants is greatly increased (see Table 5). The letters "n" and "m" designate the number of enzymatic reactions involved in MDO synthesis, which are not shown at present. A genetic symbol next to a specific reaction implies the availability of mutants at that site.

pended during formation of the fatty acid chains. Taken together, these observations imply the existence of the diglyceride cycle shown in Fig. 5, which presumably occurs in the cytoplasmic membrane. A biochemical system for the generation of MDO and diglyceride will be required to elucidate the details of the reactions involved.

ACKNOWLEDGMENTS

We thank Barbara Bachmann of the E. coli Genetic Stock Center, Yale University, for some of the strains shown in Table 1 and Roy Black for helping us set up the methods for membrane separation shown in Fig. 1. We thank Masahiro Nishijima for the electrophoretic analysis of membrane protein composition and Dan Fraenkel for helpful discussions.

This research was supported in part by Public Health Service grants AM 19551 and KO4-AM 00584 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, by Hatch Project 2269 from the Cooperative State Research Service, and by a Harry and Evelyn Steenbock Career Advancement Award to C.R.H.R.

LITERATURE CITED


Vol. 137, 1979 DIGLYCERIDE CYCLE IN E. COLI 867

Downloaded from http://jb.asm.org/ on July 8, 2017 by guest