Phosphatidyltransferase activity in *Bacillus megaterium*

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(Received 2 January 1991; accepted 18 April 1991)

Phosphatidyl transfer between phosphatidylethanolamine, phosphatidylglycerol or phosphatidylserine as donors and primary hydroxyl acceptors including ethanolamine, glycerol, serine and Triton X-100 has been shown to be catalysed by membrane particles derived from *Bacillus megaterium* strains ATCC 13632 and ATCC 14581. The rate of cardiolipin synthesis from phosphatidylglycerol in the presence of ethanolamine was an order of magnitude greater than that of phosphatidylethanolamine formation. Cardiolipin synthesis from phosphatidylethanolamine in the presence of glycerol was also observed, and was 1.5-fold greater than the formation of phosphatidylglycerol. Similar heat lability, effects of pH and of Triton X-100 for phosphatidyl transfer and cardiolipin synthesis indicate that both reactions were catalysed by cardiolipin synthase.

Introduction

The biosynthesis of phosphoglycerides in the eubacteria generally appears to follow the branched pathway described in *Escherichia coli* by Kennedy and his colleagues (reviewed by Raetz, 1978; Pieringer, 1989). In these pathways the formation of the polyglycerol phosphatides phosphatidylglycerol (PG) and cardiolipin (CL), and the pathway to phosphatidylethanolamine (PE) diverge after the conversion of phosphatidic acid (PA) to CDP-diacylglycerol, which can react either with sn-glycerol-3-phosphate to form phosphatidylglycerol-phosphate or with serine to form phosphatidylserine (PS). These in turn give rise to PG and PE.

Two laboratories have reported evidence for pathways involving interconversion of PG and PS, which can then be decarboxylated to form PE. Working with *Bacillus megaterium*, Lombardi & Fulco (1980) and Lombardi et al. (1980) presented evidence for two pools of PG, one stable and one active metabolically. Pulse-chase experiments indicated that the bulk of PE synthesized in growing cultures arose from PG and diacylglycerol. However, Langley et al. (1979) have shown that PG and PS are synthesized at similar rates in *B. megaterium*. They could detect no evidence of a base-exchange activity in extracts of these cells. It should be noted, however, that the two laboratories studied different strains of *B. megaterium*.

Yokota & Kito (1982) demonstrated the formation of PE from a pool of PG in a serine auxotroph of *E. coli*. Most of the radioactivity in PG labelled in the diacylglycerol moiety in a serine-deficient medium was transferred to PE during a chase in the presence of serine. Although a base-exchange mechanism could not be ruled out, these authors concluded that turnover of PG led to the formation of radiolabelled PE through the conventional phosphatidic acid and CDP-diacylglycerol intermediates. This conclusion was largely based on dissimilarities in the molecular species of PG and the newly formed PE.

Evidence for base exchange catalysed by a phosphatidyltransferase in *Clostridium butyricum* was provided by this laboratory (Walton & Goldfine, 1987; Morii & Goldfine, 1990). Several phospholipids, including PG, PE and PS, were shown to serve as phosphatidyl donors, and primary alcohols including serine, ethanolamine, glycerol and Triton X-100 served as acceptors in the phosphatidyltransferase-catalysed reaction. Phosphatidyl transfer to PG was also observed, but this activity could be distinguished from that of CL synthase by differences in pH optima and heat lability.

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Abbreviations: CL, cardiolipin; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PT, phosphatidyl Triton X-100.

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In view of these results, we have performed studies on extracts of \textit{B. megaterium}, which were designed to detect a similar phosphatidyltransferase activity. Although phosphatidyl transfer between phospholipids and primary alcohols, including Triton X-100, was observed, it appears that these reactions were catalysed by CL synthase. These experiments are described in the present report.

**Methods**

*Strains.* \textit{B. megaterium} ATCC 13632 and ATCC 14581 were grown at 35 °C on LB medium (tryptone 1\%, yeast extract 0-5\%, NaCl 1\%, pH 7.0), 500 ml per 2 litre flask, on a rotary shaker at approximately 200 r.p.m. Growth of the cells was monitored by turbidity measurements in a Klett colorimeter and they were harvested in the late exponential phase of growth by centrifugation at 4200 g for 10 min in a refrigerated centrifuge. Membrane particles were prepared after disrupting the cells with a French pressure cell as described previously (Walton & Goldfine, 1987). Unless otherwise indicated, membrane particles from \textit{B. megaterium} ATCC 14581 were used.

*Production of labelled phospholipids.* [\(^{32}\)P]PG was prepared from the CL-synthase-minus mutant \textit{E. coli} T1GP (Pluschke et al., 1978) and [\(^{32}\)P]PE and [\(^{32}\)P]PS were prepared from a plasmalogen-deficient strain of \textit{Megasphaera elsdii} (Verma & Goldfine, 1983; Kaufman et al., 1988) as described previously (Walton & Goldfine, 1987). The labelled phospholipids were purified by thin-layer chromatography (TLC) on silica gel 60 plates (E. Merck). The solvent systems used were: (A) chloroform/methanol/7 M-ammonium hydroxide, 60:35:5, by vol., for one-dimensional chromatography and for the first dimension of two-dimensional chromatograms; and (B) chloroform/methanol/acetic acid, 65:25:8, by vol., for the second dimension of two-dimensional chromatograms.

*Enzyme assays.* These were carried out as described previously (Walton & Goldfine, 1987) with the following additions: 20 mm-potassium phosphate buffer, pH 7-0; 15 mm-MgCl\(_2\); 4-5 mm-dithiothreitol; 1-2 mg Triton X-100 ml\(^{-1}\); 0-2 mm-substrate phospholipids (20000 c.p.m.) and 10 mm-acceptors, unless otherwise indicated, in a final reaction volume of 0.2 ml. \textit{B. megaterium} membrane particles were added to start the reaction. Two hundred micrograms of membrane protein contained 26 nmol PG and 20 nmol PE, which were determined as described previously (Morii & Goldfine, 1990). Calculations of product formation included the amount of substrates contributed by the membrane particles. Incubation was for 30 min unless otherwise indicated, at 37 °C. Extraction and chromatography of the products of enzyme reactions were carried out essentially as described previously (Walton & Goldfine, 1987).

*Other analytical methods.* Mild alkaline deacylation of phospholipids to yield the water-soluble glycerophosphoryl esters was performed as described by Kates (1972). Identification of the deacylation products was based on TLC on cellulose TLC plates (E. Merck) with solvent (C), ethanol/1 M-ammonium acetate, pH 7-5, 65:35, v/v, as described previously (Walton & Goldfine, 1987). Protein was determined by the Lowry method.

*Materials.* Bovine brain PS, \textit{E. coli} PE, PG (made from egg yolk phosphatidylcholine), bovine heart CL, dipalmitoylphosphatidic acid, Triton X-100, ethanolamine, glycerol and serine were obtained from Sigma. Solvents were glass-distilled (Burdick & Jackson, Muskegan, MI, USA). All other reagents were analytical grade.

![Time course of formation of PE (\(\square\)), PT (\(\bullet\)) and PA (\(\square\)) from \(^{32}\)P]PG. The standard assay conditions were used with 400 μg membrane particles. The curve is representative of data from several experiments.](image)

**Results**

*Identification of products.*

The products formed from [\(^{32}\)P]PG in the presence of ethanolamine were identified as PE, CL, PA and phosphatidyl Triton X-100 (PT) and the products formed from [\(^{32}\)P]PE in the presence of free glycerol were identified as PG, CL and PA. These products were scraped and eluted from the silica gel after one-dimensional TLC in solvent A. With the exception of PT, each of these radioactive lipid products was shown to co-chromatograph with authentic standards in the two-dimensional TLC system. In addition, each lipid was subjected to mild alkaline deacylation and the radiactive water-soluble product was chromatographed on cellulose TLC and shown to have the same \(R_F\) as the deacylation products obtained from authentic lipid standards, as described previously (Walton & Goldfine, 1987). Decayed PT, prepared from [\(^{32}\)P]PG with membrane particles from \textit{B. megaterium}, comigrated in the two-dimensional TLC system with deacylated PT prepared by a similar incubation with membrane particles from \textit{C. butyricum} (Walton & Goldfine, 1987) (data not shown).

*Characterization of the transphosphatidylation reaction.*

The formation of PE from [\(^{32}\)P]PG and ethanolamine was linear with respect to protein concentration up to 600 μg per assay (3-0 mg/ml\(^{-1}\)), and 200 to 400 μg protein per assay was used in the standard incubation mixture. The kinetics of product formation are illustrated in Fig. 1. The formation of PE was usually linear for 30 min, and the kinetics of formation of PT and PA were variable (Fig. 1). Concentration-dependence curves were deter-
transphosphatidylation activities of the two strains of *B. megaterium* which are given in Table 1. In general, comparable activities were seen with membrane particles derived from strains ATCC 13632 and ATCC 14581. Divalent cations did not appear to be required for activity. The enzyme was insensitive to 10 mM EDTA, and 20 mM Ca$^{2+}$, Zn$^{2+}$ and Mn$^{2+}$ were inhibitory to the formation of both PE and PT from [$^{32}$P]PG and ethanolamine (data not shown).

As shown in Fig. 2, a large amount of CL was also formed when PG was employed as the phosphatidyl donor. The velocity of CL formation was 1.46 ± 0.05 nmol min$^{-1}$ (mg protein)$^{-1}$ (n = 4), about twelve times as great as that of PE in the presence of added ethanolamine. As noted above, the formation of

![Graph](image)

**Fig. 3.** Thermal inactivation profiles. Membrane particles from *B. megaterium* ATCC 14581 were preincubated at 50 °C or 55 °C for the indicated times. Preincubation was stopped by rapidly chilling the particles on ice. Assays were performed under the standard incubation conditions with 200 μg membrane protein, [$^{32}$P]PG and ethanolamine. Products of the reaction were CL (○, preincubation at 50 °C; ●, preincubation at 55 °C) and PE (□, preincubation at 50 °C; ■, preincubation at 55 °C). Without heating, the rate of CL formation was 2.0 nmol min$^{-1}$ mg$^{-1}$ and the rate of PE formation was 0.088 nmol min$^{-1}$ mg$^{-1}$. The data for heating at 50 °C are representative of two experiments. The data for heating at 55 °C represent the means ± SE of two determinations.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Donor</th>
<th>Serine</th>
<th>Ethanolamine</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.012 (1)</td>
<td>0.014 ± 0.008 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCC 13632</td>
<td>-</td>
<td>0.058 (1)</td>
<td>0.009 ± 0.003 (2)</td>
</tr>
<tr>
<td></td>
<td>ATCC 14581</td>
<td>-</td>
<td>0.0024 ± 0.0011 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCC 13632</td>
<td>ND</td>
<td>-</td>
<td>0.025 ± 0.005 (2)</td>
</tr>
<tr>
<td></td>
<td>ATCC 14581</td>
<td>0.028 (1)*</td>
<td>0.124 ± 0.015 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCC 13632</td>
<td>0.033 (1)</td>
<td>0.24 (1)</td>
<td>-</td>
</tr>
</tbody>
</table>

*ND*, Not detectable.

*An additional measurement with a 60 min incubation gave an identical value.*
Fig. 4. Effects of pH on the formation of CL (○) and PE (■) in vitro from [32P]PG. Assays were performed under the standard conditions, with 200 μg membrane protein, in the presence of 50 mM-MES buffer at the indicated pH. The curves represent the means of two determinations.

Table 2. Effects of Triton X-100 and p-hydroxymercuribenzoate on the formation of CL and PE from [32P]PG and ethanolamine

<table>
<thead>
<tr>
<th>Addition to assay mixture</th>
<th>Amount formed (nmol per assay)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL</td>
</tr>
<tr>
<td>None</td>
<td>5.93 (100)</td>
</tr>
<tr>
<td>Triton X-100</td>
<td></td>
</tr>
<tr>
<td>0.5%</td>
<td>0.68 (11.5)</td>
</tr>
<tr>
<td>1.0%</td>
<td>0.04 (0.7)</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td></td>
</tr>
<tr>
<td>0.05 mM</td>
<td>5.70 (96)</td>
</tr>
<tr>
<td>0.10 mM</td>
<td>4.86 (82)</td>
</tr>
<tr>
<td>0.05 mM</td>
<td>5.30 (89)</td>
</tr>
</tbody>
</table>

* The percentage of the control assay is shown in parentheses.

PE from PG was dependent on the presence of ethanolamine. There was no difference in CL formation in the presence and absence of ethanolamine (Fig. 2). When PE was used as a phosphatidyl donor in the presence of glycerol, cardiolipin formation was 1.5 times greater than PG formation, but the rate of CL synthesis was 3.9-fold lower than that seen when PG was employed as the phosphatidyl donor [0.037 vs 1.46 nmol min⁻¹ (mg protein)⁻¹].

Thermolability, effects of inhibitors and pH optimum

The ability to form both CL and PE from PG with ethanolamine present was resistant to heating at 50 °C, and both activities had essentially identical thermal lability profiles at 55 °C (Fig. 3). p-Hydroxymercuribenzoate is an inhibitor of CL synthase in Lactobacillus plantarum at 0.1 mM (Burritt & Henderson, 1975). No inhibition by 0.2 mM-p-hydroxymercuribenzoate was seen for either CL or PE formation from PG in reactions catalysed by B. megaterium membrane particles. Transphosphatidylation required Triton X-100. The optimal concentration was 0.1 to 0.2%, but the detergent was inhibitory at concentrations of 0.5% and above. These results are summarized in Table 2. The formation of CL had a slightly more acidic pH optimum than the formation of PE from PG (Fig. 4).

Discussion

Membrane particles of B. megaterium catalyse the formation of PE from PG in the presence of Triton X-100 and ethanolamine. Low transphosphatidylation activities were also observed with PS as phosphatidyl donor and ethanolamine or glycerol as acceptors and with PE as donor and serine or glycerol as acceptors (Table 1). With the exception of transphosphatidylation from PG to ethanolamine, the initial velocities of these reactions were at least an order of magnitude lower than those seen with membrane particles from C. butyricum (Walton & Goldfine, 1987). V_max with PG as donor and ethanolamine as acceptor was 61% of that observed in experiments with C. butyricum membrane particles. Unlike the phosphatidyltransferase activity from C. butyricum, that from B. megaterium was stable to heating at 50 °C, as was the formation of CL from PG. CL synthesis activity with C. butyricum membrane particles was stable to heating at 45 °C, whereas the phosphatidyltransferase was rapidly inactivated at that temperature (Walton & Goldfine, 1987). The pH optimum for PE formation from PG and ethanolamine was slightly more basic than that for CL synthesis from PG, but both activities were similarly increased at pH 7.0 and 5.5 (Fig. 4). Both activities were similarly affected by Triton X-100 addition, and neither was inhibited by p-hydroxymercuribenzoate. The formation of PE and PT from PG was inhibited by divalent cations at 10 to 20 mM. In addition to direct effects on the enzyme, this may have resulted from complex formation and withdrawal of the substrate or from sequestering of acidic lipids in the cell membrane.

Although these results do not permit a definitive distinction between CL synthase and phosphatidyltransferase activities in B. megaterium, it seems likely that...
Transphosphatidylation is catalysed by CL synthase, which is at least tenfold more active than the transphosphatidylation reactions observed with these membrane particles. However, these experiments do reveal several apparent phosphatidyl transfer activities, including the formation of PT. Pulse-chase experiments with B. megaterium in vivo provided evidence for the use of a pool of PG for PS and PE synthesis (Lombardi & Fulco, 1980; Lombardi et al., 1980). Formation of a phosphatidyl-carrier intermediate with transfer to serine was postulated based on several lines of evidence obtained with whole cells. Our experiments revealed comparable transphosphatidylation activities in the strains studied by Lombardi et al. (1980) and by Langley et al. (1979), who reached different conclusions (see Introduction). Furthermore, transphosphatidylation from PG to serine was four- to sevenfold slower than transphosphatidylation to ethanolamine (Table 1). Further work is required to rule out completely the presence of an independent phosphatidyltransferase in B. megaterium. The activities observed could result from the formation of a phosphatidyl-enzyme intermediate in CL synthesis and the transfer of the phosphatidyl moiety to alternative acceptors such as water, soluble primary alcohols and amphiphiles such as Triton X-100. Membrane extraction and purification of the CL synthase would represent a logical extension of this work to determine whether separate enzymes catalyse these activities in B. megaterium. Recent work with E. coli has shown the formation of unknown lipids upon addition of high levels of certain sugars to the growth medium. Among the unusual lipids formed were phosphatidylmannitol and the CL analogue diphosphatidylmannitol. Formation of these analogues was not observed in a CL synthase mutant. It was concluded that the CL synthase of E. coli is sufficiently nonspecific to permit alcoholysis of CL to yield PG and the phosphatidyl sugar, which subsequently recondensed to form the corresponding diphosphatidyl sugar (Shibuya et al., 1985). Having similar CL synthase mutants of B. megaterium would also help to distinguish among the various possibilities discussed above.

The biological significance of transphosphatidylation in B. megaterium and in other bacteria is uncertain at present. We have presented evidence for donor specificity in C. butyricum (Morii & Goldfine, 1990). It is not known if the intracellular concentrations of water-soluble acceptors such as ethanolamine or glycerol are sufficiently high for transphosphatidylation to these acceptors to occur in either species.

This work was supported by Public Health Service Research Grant, AI-08903, from the National Institute of Allergy and Infectious Diseases.

References


