THE ROLE OF PHOSPHATIDYLGLYCEROL IN THE IN VITRO BIOSYNTHESIS OF TEICHOIC ACID AND LIPOTEICHOIC ACID

Larry EMDUR and Teh-hsing CHIU
Department of Microbiology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, 15261, USA

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1. Introduction

The biosynthesis of glycerol teichoic acid was originally reported by Burger and Glaser [1] to involve CDP-glycerol as the donor of glycerolphosphate moieties in *Bacillus licheniformis* and *Bacillus subtilis*. Based on indirect evidence, Anderson et al. [2] suggested the involvement of glycosylpolyisoprenylphosphate intermediate in the biosynthesis of teichoic acid. However, the in vivo pulsechase experiments done in our laboratory [3,4] and Glaser's laboratory [5] suggested that phosphatidylglycerol [PG] is the precursor in lipoteichoic acid biosynthesis. In this communication direct evidence is presented to show that PG could serve as donor of the glycerol and phosphate moieties in teichoic acid and lipoteichoic acid biosyntheses. Fatty acid moieties of lipoteichoic acid may also be derived from PG.

2. Materials and methods

The sources of *S. sanguis* and chemicals were the same as previously reported [6]. [2-^{3}H]Glycerol (2 mCi/μmol), [1-^{14}C]acetate (25-60 Ci/μmol) and [^{32}P]-phosphate (28 Ci/μmol) were obtained from International Chemical and Nuclear Corp. and [^{14}C]UDP-glucose (227 Ci/μmol) from New England Nuclear. Thin-layer chromatography was performed on Absorbosil-5 thin-layer plates (0.25 mm thick) (Applied Science), developed in hexane:ethyl ether: acetic acid (50:50:1.5) (solvent A) or in acetone: petroleum ether (1:3), dried and redeveloped in chloroform:methanol:acetic acid:water (80:13:8:0.3) (solvent B). Alkaline Absorbosil-5 thin-layer plates [7] were developed in chloroform:methanol:1 M ammonium hydroxide (80:36:2) (solvent C). Paper chromatography was carried out on Whatman 3MM paper and developed in pyridine:water:ethyl acetate: acetic acid (5:3:5:1) (solvent D) or butanone:acetic acid:water (75:25:10) (solvent E). Radiolabeled and reference compounds on chromatograms were detected as described previously [4].

Radioisotope labeled PG was isolated from the chloroform:methanol extract of a mid-log phase *S. sanguis* culture which was pulsed for 20 min with 2 mCi of [2-^{3}H]glycerol, [^{32}P]phosphate or [1-^{14}C]acetate [4]. The crude lipid extract was fractionated either by silicic acid column chromatography into neutral lipid, glycolipid and phospholipid [PG] [8] or by diethylaminoethyl [DEAE]-cellulose column chromatography in which PG was eluted by 7.5 mM ammonium acetate and phosphatidic acid was eluted by 50 mM ammonium acetate. The PG prepared by both procedures co-chromatographed on thin layer plates with authentic PG (Serdary Research Lab.) in solvents B and C. When [^{3}H] or [^{32}P]PG was subjected to mild alkaline hydrolysis [6] and chromatographed on Whatman 3MM paper, at least 95% of the radioactive material exhibited the same mobility as that of glycerolphosphorylglycerol in solvents D and E. The particulate enzyme was prepared from *S. sanguis* according to Strominger et al. [9].

3. Results and discussion

The incorporation of ^{3}H from [^{3}H]PG into the NaCl extract and residue fractions was linear during the 75 min assay (fig.1). Slightly more ^{3}H was

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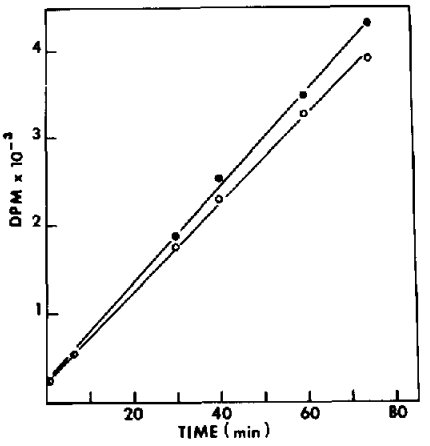

Fig.1. Kinetics of transfer of [^{3}H]glycerol from [^{3}H]PG into the NaCl extract and residue. [^{3}H]PG (1.3 × 10^5 dpm) was dried in 5 ml conical tubes, placed in a dessicator and evacuated for 2 h to remove organic solvent. Tris–(hydroxymethyl) aminomethane–HCl (tris–HCl), 150 mM, pH 8.0 containing 32 mM Mg^{++}, 0.2 mM 2-mercaptoethanol (TMM buffer) in a total vol of 250 μl was added to the dried lipid. The reaction mixture was then sonicated twice for 20 sec using a Sonifier Cell Disruptor equipped with a 3 mm micro-tip disruptor horn in order to disperse the lipid. 200 μg of particulate enzyme and 4 mM UDP-glucose was added to the reaction mixture which was then incubated with shaking at 37°C. At times indicated, the reaction mixtures were extracted twice with 12 vol of chloroform–methanol (1:1). The precipitate was extracted twice with 0.85% NaCl. The combined NaCl extracts were dried in scintillation vials and counted. The remaining residue was suspended in toluene-Triton X-100 scintillation fluid and also counted (residue). (○——○) residue; (●——●) NaCl extract.

incorporated into the NaCl extract than the residue. Seventy-eight percent of the radioactivity in the NaCl extract was nondialyzable. When UDP-glucose was deleted from the reaction mixture, the rate of incorporation of ^{3}H into the NaCl extract and residue was approximately 50% less. Addition of 1 mM PG or cardiolipin to the reaction mixture inhibited the incorporation into the NaCl extract by 48 and 68%, respectively. Similar results were obtained when [^{32}P]PG was used as substrate. No significant incorporation of radioactivity was observed when [^{3}H]neutral lipid, [^{3}H]glycolipid or [^{3}H]phosphatidic acid were used instead of [^{3}H]PG. Large scale reactions were carried out to obtain sufficient material for the characterization of products. Two radioisotope labeled substrates were used (e.g. either [^{3}H, ^{14}C]PG, [^{14}C, ^{32}P]PG or [^{3}H]PG and [^{14}C]UDP-glucose). The results described below are obtained from one experiment where 2.5 ml of TMM buffer was added to a conical tube containing 10 mg particulate enzyme, 3.85 μCi of [^{3}H]PG, 2 μCi of [^{14}C]PG and 8 mM UDP-glucose. The tube was incubated as described in fig.1. After 90 min incubation, the reaction mixture was extracted twice with twenty volumes of chloroform: methanol (1:1) (C:M Extract) and the residue was extracted twice with 4 ml of 0.85% NaCl (NaCl extract). The remaining residue was suspended in 5 ml of water (residue). Nondialyzable radioactive material was found in each of the above three fractions. The C:M and NaCl extracts were purified and characterized as described below: Approximately 9% of the ^{3}H and 0.32% of the ^{14}C from [^{3}H, ^{14}C]PG was incorporated into the nondialyzable NaCl extract. This material (8 × 10^5 dpm of [^{3}H] and 1.4 × 10^4 dpm of ^{14}C)

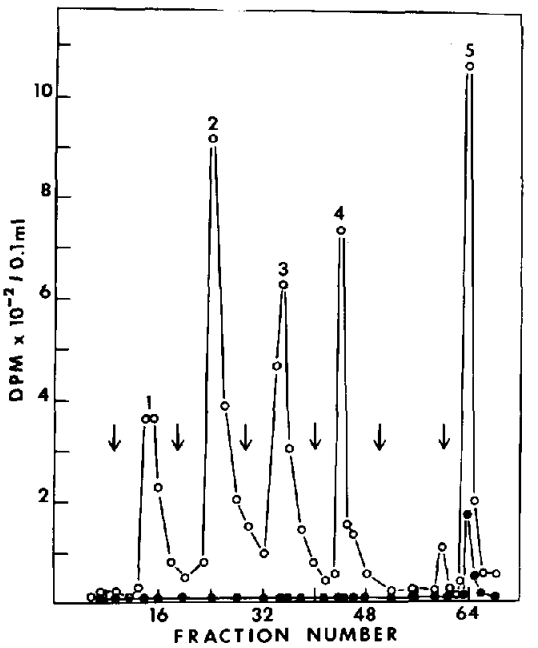

Fig.2. Ecteola-cellulose column chromatographic purification of the NaCl extract. The dialyzed NaCl extract was loaded onto a Ecteola-cellulose column (0.7 × 15 cm) equilibrated with distilled water. The column was washed with water and eluted stepwise consecutively at arrows with 0.05 M, 0.1 M, 0.15 M, 0.5 M, 1 M LiCl and 1 M LiCl containing 1% Triton X-100. 1.5 ml fractions were collected at a rate of 0.3 ml/min. (○) ^{3}H; (●) ^{14}C.

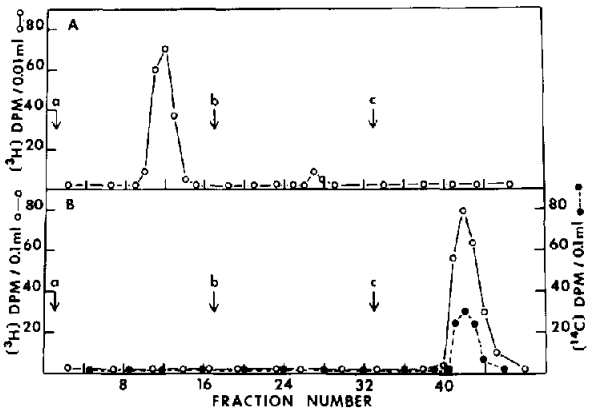

Fig.3. (A) Con A column chromatographic purification of peak 2. The 0.1 M LiCl eluate (peak 2) from the previous Ecteola-cellulose column was dialyzed overnight (1.92×10^5 dpm of [^{3}H]) and loaded onto a Con A column (0.7×15 cm) equilibrated with 0.02 M Tris–HCl, pH 7.5. The column was eluted with 0.02 M Tris–HCl (a), 0.02 M Tris–HCl–0.1 M α-methylglucoside (b), 0.02 M Tris–HCl–0.1 M α-methylglucoside–1% Triton X-100 (c). Fractions of 0.8 ml were collected. (B) Con A column chromatographic purification of peak 5. The 1 M LiCl–1% Triton X-100 eluate (peak 5) from the previous Ecteola-cellulose column was extracted 8 times with toluene to remove Triton X-100 and dialyzed. The dialysate was concentrated under reduced pressure to 2 ml (^{3}H), 4.6×10^4 dpm; ^{14}C, 7.6×10^3 dpm) and was loaded onto a Con A column (0.7×15 cm) and eluted as was described above. (○) ^{3}H; (●) ^{14}C.

was fractionated into five peaks by Ecteola-cellulose column chromatography (fig.2). Sixty percent of the ^{3}H and 54% of the ^{14}C were recovered. The amount of ^{3}H incorporated into peaks 1, 2, 3, 4 and 5 from PG was 0.7, 2, 1.4, 0.5 and 0.5%, respectively. All of the [^{14}C]fatty acid in NaCl extract was associated with peak 5. In an identical experiment in which [^{14}C]UDP-glucose and [^{32}P]PG were used in the reaction mixture, ^{14}C and ^{32}P were found to be associated with each peak. Peak 2 was separated into two components by Concanavalin A-Sepharose 4B affinity column (Con A column) chromatography; one component was eluted by buffer a (2A) (e.g. 82% of the input) and the other by buffer b (2B) (e.g. 4% of the input) (fig.3A). Peak 5 was eluted as a single peak from the same column by buffer c (5C) (fig.3B). A total recovery of 40% each of ^{3}H and ^{14}C was obtained in this last column. The ^{14}C in 5C was present as [^{14}C]fatty acids since transesterification of 5C with toluene.

methanol:KOH (5:14:1) [6] released [^{14}C]methyl esters which co-chromatographed on thin-layer plates in solvent A with methyl palmitate. Acid hydrolysis of 2A, 2B and 5C released compounds with the paper chromatographic mobilities in solvent D of glycerol, α-glycerolphosphate and glycerolphosphate, respectively. When [^{14}C]UDP-glucose and [^{32}P]PG were used as the radioactive substrates in the reaction mixture, [^{14}C]glucose and [^{32}P]α-glycerolphosphate and glyceroldiphosphate were detected in the acid hydrolysates of 2A, 2B and 5C. 2A and 2B are two forms of polyglycerolphosphate in which 2B contains more glucose moieties than 2A. We propose that peak 5C is a lipoteichoic acid on the basis of its column chromatographic behavior (figs.2, 3B) and the presence of glycerol, phosphate, glucose and fatty acid. These results suggest that teichoic acid and lipoteichoic acid were synthesized in vitro and that glycerol and phosphate were derived from PG.

A [^{3}H, ^{14}C]polymer was also found to be extracted into the C:M extract. At least 1.5% of the radioactivity from [^{3}H, ^{14}C]PG was incorporated into this polymer which was characterized as a lipoteichoic acid since its column chromatographic behavior was similar to 5C (figs. 2, 3B) and it was composed of glycerol, phosphate, glucose and fatty acids. The procedure used to isolate the lipoteichoic acid in the C:M extract will be published elsewhere.

The residue glycerolpolymer has not yet been characterized. Approximately 4% of the radioactivit from [^{3}H]PG was incorporated into this polymer.

Optimum conditions for the biosynthesis of the polymers was found to be 32 mM in Mg^{++} and a pH of 8.0. The reaction was linearly dependent on the concentration of phosphatidylglycerol and enzyme. If exogenous UDP-glucose was deleted from the reaction mixture, only polyglycerolphosphate was detected in the NaCl extract.

In summary, a particulate enzyme from *S. sanguis* catalyzed the synthesis of three glycerolphosphate-containing polymers from PG. A total of 10.6% of the ^{3}H from [^{3}H]PG was incorporated into glycerol teichoic acid (4.6%), lipoteichoic acid (2%) (0.5% in the NaCl extract and 1.5% in the C:M extract) and residue glycerolpolymer (4%). PG may have served as a donor of the fatty acid moieties of lipoteichoic acid. Whether the lipoteichoic acid or the residue glycerolpolymer is equivalent to the lipoteichoic

acid carrier reported by Fiedler and Glaser [10] is not known. These present findings confirm our in vivo study demonstrating that PG is a precursor of teichoic acid and lipoteichoic acid [3,4]. These results are in contrast to those of Burger and Glaser [1] who reported earlier that CDP-glycerol is the precursor of glycerol teichoic acid in *B. licheniformis* and *B. subtilis*.

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