Phosphatidylglycerol as biosynthetic precursor for the poly(glycerol phosphate) backbone of bifidobacterial lipoteichoic acid

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Phosphatidylglycerol functions as donor of the *sn*-glycerol 1-phosphate units in the synthesis *in vitro* of the 1,2-phosphodiester-linked glycerol phosphate backbone of the lipoteichoic acids of *Bifidobacterium bifidum* subsp. *pennsylvanicum*. The incorporation was catalysed by a membrane-bound enzyme system. After addition of chloroform/methanol the product formed coprecipitated with protein. The material was phenol-extractable and was co-eluted with purified lipoteichoic acid on Sepharose 6B. The reaction was stimulated by Triton X-100, UDP-glucose and UDP-galactose, but Mg^{2+} ions had no effect. The apparent values for K_m and V_{max} of the phosphatidylglycerol incorporation were 1.4 mM and 3.1 nmol/h per mg of membrane protein, respectively. Labelled UDP-glucose and UDP-galactose were not incorporated into the lipoteichoic acid fraction by the particulate membrane preparation.

The lipoteichoic acid molecules of *Bifidobacterium bifidum* subsp. *pennsylvanicum* consist of linear 1,2-phosphodiester-linked glycerol phosphate backbones substituted at the terminal glycerol with a polysaccharide containing either galactose or glucose (Op den Camp *et al.*, 1984). The other end of the glycerol phosphate polymer is covalently linked to 3-*O*-[6'-(*sn*-glycero-1-phosphoryl)diacyl- β -D-galactofuranosyl]-*sn*-1,2-diacylglycerol. After studying some biological properties of this rather unique amphiphile (Op den Camp *et al.*, 1985*a,b*) we initiated a study of its biosynthesis.

Fischer (1980) reviewed metabolic and structural evidence that glycerophosphoglycolipids of many Gram-positive bacteria are related to the lipoteichoic acids. Previously (Veerkamp, 1976; Van Schaik & Veerkamp, 1976), we reported the synthesis of lipid 6, the lipid anchor of the bifidobacterial lipoteichoic acid, by transfer of *sn*-glycerol 1-phosphate from phosphatidylglycerol to the related galactolipid. Studies concerning the biosynthesis of lipoteichoic acids (Emdur & Chiu, 1974, 1975; Ganfield & Pieringer, 1980; Glaser & Lindsay, 1974; Koga *et al.*, 1984) have given evidence that the glycerol phosphate moieties of these molecules also originate from phosphatidylglycerol. The unacylated *sn*-glycerol 1-phosphate of the lipid anchor represents the first unit of

the lipoteichoic acid backbone (Fischer, 1980; Ganfield & Pieringer, 1980).

Since pulse-labelling experiments *in vivo* with [^{14}C]glycerol were not possible with *B. bifidum* due to the absence of glycerol kinase (Molenkamp & Veerkamp, 1976) we studied lipoteichoic acid synthesis *in vitro* using a particulate enzyme preparation of this organism. Studies revealed that the *sn*-glycerol 1-phosphate unit from phosphatidylglycerol is incorporated into the bifidobacterial lipoteichoic acid. The role of the lipid anchor and of UDP-glucose and UDP-galactose in this process has also been investigated.

Experimental

Materials

CDP-diacylglycerol was obtained from Serdary Research Laboratories (London, Ontario, Canada). Lysozyme (EC 3.2.1.17), DNAase (EC 3.1.4.5), CTP, UDP-glucose and UDP-galactose were purchased from Boehringer (Mannheim, Germany); labelled compounds were from Amersham International. All other chemicals were of analytical grade.

Growth of bacteria and preparation of bacterial fractions

Bifidobacterium bifidum subsp. *pennsylvanicum* was cultivated in 800 ml batches for 16 h under the conditions described previously (Op den Camp *et*

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al., 1984). Lipoteichoic acid was isolated from disintegrated bacteria and purified by column chromatography (Op den Camp *et al.*, 1984). A particulate membrane fraction was prepared by lysis of the bacteria with lysozyme, treatment with DNAase and ultracentrifugation (Veerkamp, 1974). The protein content of the particulate fraction was $54 \pm 2\%$ (mean $\pm$ S.D. for four preparations) as determined by the method of Lowry *et al.* (1951).

Preparation of labelled precursors

Phosphatidyl[^{14}C]glycerol (sp. radioactivity $0.05 \mu\text{Ci}/\mu\text{mol}$) was synthesized from *sn*-[1- ^{14}C]glycerol 3-phosphate and CDP-dioleoylglycerol and purified as described by Veerkamp (1976). CDP-[^{14}C]glycerol was prepared from *sn*-[1- ^{14}C]glycerol 3-phosphate and CTP (Veerkamp, 1976). [^{14}C]Oleic acid-labelled lipids were obtained by cultivation of *B. bifidum* subsp. *pennsylvanicum* in the presence of [^{14}C]oleic acid ($250 \mu\text{Ci/l}$) and a Tween-80 concentration of only 0.1 g/l . The labelled lipids were fractionated on a silica gel column (Exterkate & Veerkamp, 1969) to separate glyco- and phosphoglyco-lipids.

Incorporation experiments

Incorporation of phosphatidyl[^{14}C]glycerol was performed using the assay mixture described by Ganfield & Pieringer (1980) with some modifications. Briefly, the indicated amount of phosphatidyl[^{14}C]glycerol in chloroform/methanol (2:1, v/v) was transferred into the test tube. The solvent was removed by a stream of N_2 . After addition of $75 \mu\text{l}$ of 50 mM -Tris/HCl assay buffer (pH 7.0), containing 0.1% Triton X-100, 5 mM -UDP-glucose and 5 mM -UDP-galactose, the mixture was sonicated for 1 min in an ultrasonic waterbath (Bransonic B12). The reaction was started by the addition of about $750 \mu\text{g}$ of particulate fraction ($400 \mu\text{g}$ of protein) suspended in $50 \mu\text{l}$ of the same buffer. The mixture was usually incubated at 37°C for 4 h.

The incorporation was terminated with the addition of 2 ml of chloroform/methanol (1:1, v/v). The protein was pelleted by centrifugation and washed once with 2 ml of chloroform/methanol. The supernatants were combined and extracted with 1 ml of 0.2% NaCl. Saline-extractable radioactivity was determined by liquid-scintillation counting in OptiFluor (Packard). The pellet was suspended in 0.5 ml of 0.1 M -acetate buffer (pH 5.5) containing 20 mM - MgSO_4 and extracted with 0.5 ml of 80% (w/v) phenol for 30 min at 60°C . The aqueous layer was collected by centrifugation ($10000g$, 10 min) and the organic layer was washed with 0.5 ml of the acetate buffer. The aqueous layers were collected (lipoteichoic acid fraction) and the incorporated radioactivity was measured.

We also determined the radioactivity remaining in the phenol residue.

In some experiments [^{14}C]oleic acid-labelled phosphoglycerolipids, UDP-[^{14}C]glucose or UDP-[^{14}C]galactose replaced phosphatidylglycerol as the radioactive substrate. In these experiments 100 nmol of unlabelled phosphatidylglycerol was added to each test tube. The incorporation of UDP-[^{14}C]glucose or UDP-[^{14}C]galactose into the bifidobacterial lipoteichoic acid was also tested by using the assay system described by Arakawa *et al.* (1981), with $7.5 \mu\text{g}$ of lipoteichoic acid added to each test tube.

Identification of the radioactive products

The saline extract of the supernatant and the phenol extract of the pellet (lipoteichoic acid fraction) were washed twice with equal volumes of chloroform, concentrated and applied to a Sepharose 6B column ($1.7 \times 50 \text{ cm}$). The column was eluted with 0.2 M -ammonium acetate (pH 7.0) containing 0.02% NaN_3 . Fractions (5.5 ml) were collected and assayed for radioactivity. The peaks containing radioactivity were further treated as described under 'Results'. Strong alkaline hydrolysis and identification of products by paper electrophoresis were as previously described (Op den Camp *et al.*, 1984). T.l.c. was performed on silica gel 60, developed in propan-1-ol/pyridine/water (7:4:3, by vol.; system A).

Results

Characteristics of the biosynthetic reaction

The radioactivity from phosphatidyl[^{14}C]glycerol appeared to be incorporated into a product that coprecipitated with protein after addition of chloroform/methanol and that could be extracted with aqueous phenol. The residual phenol layer contained only very small amounts of radioactivity. Extraction of the pellet with 0.2% NaCl (Ganfield & Pieringer, 1980) appeared to be incomplete. The radioactivity incorporated into the saline extract of the supernatant represents mainly [^{14}C]glycerol which is liberated during synthesis of diphosphatidylglycerol from two phosphatidylglycerol molecules (Veerkamp, 1976; Van Schaik & Veerkamp, 1976). This was confirmed by t.l.c. in system A. No products with a high M_r were found in the saline fraction. The incorporation into the phenol extract was stimulated by Triton X-100 (optimal concentration 0.1%) and by UDP-glucose and UDP-galactose, when both compounds were present (Table 1). Mg^{2+} did not affect the incorporation into the phenol extract but increased the release of [^{14}C]glycerol into the saline extract of the supernatant. CDP-glycerol did not inhibit the incorporation reaction up to 100 nmol . Radioacti-

Table 1. *Effect of various factors on incorporation of phosphatidylglycerol into phenol-extractable polymer*
An amount of 180nmol of phosphatidyl[¹⁴C]glycerol was incubated with 750 μg of membrane fraction in 50mM-Tris/HCl (pH 7.0) with the indicated additions in a total volume of 125 μl. Values are means ± s.d. for three experiments. Concentrations of factors were: Triton X-100, 0.1%; UDP-glucose and UDP-galactose, 5mM.

Addition	Incorporation	
	(nmol of phosphatidylglycerol/h per mg of protein)	(%)
None	0.71 ± 0.05	100
Triton X-100	1.76 ± 0.07	248
UDP-glucose + UDP-galactose	1.37 ± 0.08	193
Triton X-100 + UDP-glucose	1.73 ± 0.05	244
Triton X-100 + UDP-galactose	1.86 ± 0.04	262
Triton X-100 + UDP-glucose + UDP-galactose	2.52 ± 0.20	355

Fig. 1. *Time course of incorporation of phosphatidyl[¹⁴C]-glycerol into the lipoteichoic acid-containing fraction*
The reaction mixture contained 180nmol of phosphatidyl[¹⁴C]glycerol and 750 μg of particulate fraction in a total volume of 125 μl of assay buffer. At the times indicated the reaction was stopped by addition of 2ml of chloroform/methanol (1:1, v/v). Other details are described under 'Experimental'. Values are means for three experiments with a variation coefficient of less than 6%. Incorporation under standard conditions (i.e. after 4h) was 2.08 ± 0.12 nmol of phosphatidylglycerol/h per mg of protein.

Fig. 2. *Enzyme-dependence of the incorporation of phosphatidyl[¹⁴C]glycerol into the lipoteichoic acid-containing fraction*
The amounts of protein indicated were incubated with 180nmol of phosphatidyl[¹⁴C]glycerol in a total volume of 125 μl of assay buffer. Other details are described under 'Experimental'. Values are means for three experiments with a variation coefficient of less than 6%. Incorporation under standard conditions was 1.76 ± 0.11 nmol of phosphatidylglycerol/h per mg of protein.

vity from CDP-[¹⁴C]glycerol (100nmol/incubation), instead of phosphatidylglycerol, was not incorporated into the lipoteichoic acid fraction. The incorporation appeared to be linear for at least 1 h (Fig. 1). For the other experiments we used an incubation time of 4 h to obtain a higher yield of incorporated radioactivity in the phenol-extractable product. Under standard conditions (400 μg of protein and 180nmol of phosphatidyl[¹⁴C]glycerol in 125 μl of assay buffer; 4 h at 37°C) we found an incorporation of 2.22 ± 0.53 nmol of phosphatidylglycerol/h per mg of protein (*n* = 12; values

from Table 1, Figs. 1, 2 and 4) with four different membrane preparations. The incorporation was proportional to the amount of membrane fraction up to 800 μg of protein (Fig. 2) and maximal within the pH range of 6–7 at 37°C.

Identification of the phenol-extractable product (lipoteichoic acid fraction)

In a large-scale incorporation experiment we incubated phosphatidyl[¹⁴C]glycerol (1 × 10⁶ d.p.m.) with 7.5 mg of particulate fraction for 4 h at 37°C in a total volume of 1.25 ml of assay buffer. The phenol extract of the pellet was applied to a Sepharose 6B column. The elution profile is shown in Fig. 3. Of the incorporated radioactivity, 79%

Fig. 3. Chromatography on Sepharose 6B of the phenol-extractable product that coprecipitated with protein. Elution was performed with 0.2M-ammonium acetate (pH 7.0), containing 0.02% NaN_3 . Fractions (5.5 ml) were collected and tested for radioactivity. V_0 , 39 ml; V_{LTA} , 60 ml, where LTA is purified lipoteichoic acid.

was eluted in a peak with an elution volume identical with that of purified lipoteichoic acid (Op den Camp *et al.*, 1984). Some residual radioactivity was eluted at the void volume and at the exclusion volume of the column. The mean peak (fractions 9–14) were pooled, dialysed against water and concentrated; 95% of the radioactivity appeared to be non-dialysable. A part of the isolated product was hydrolysed with 1M-NaOH for 1 h at 100°C and analysed by paper electrophoresis. A spot identified as glycerol phosphate contained 60% of the radioactivity applied. Some 10% of the radioactivity was found in a slow-moving compound identical with that described in the alkaline hydrolysate of lipoteichoic acid (Op den Camp *et al.*, 1984) and 30% of the label remained at the origin and could be identified as glycerol by t.l.c. in system A. From the results we may conclude that the radioactivity from phosphatidyl[^{14}C]glycerol coprecipitating with protein and extractable with aqueous phenol had been incorporated into lipoteichoic acid.

Determination of apparent K_m and V_{max} values

In order to determine the apparent K_m and V_{max} values for the incorporation of phosphatidyl[^{14}C]glycerol into the bifidobacterial lipoteichoic acid we measured the incorporation into the phenol extract with different amounts of phosphatidyl[^{14}C]glycerol per incubation (50–450 nmol). The results are shown in Fig. 4. From the Lineweaver-Burk plot (inset of Fig. 4) the apparent K_m for phosphatidylglycerol was calculated to be $1.4 \pm 0.1 \text{ mM}$ and the V_{max} , $3.2 \pm 0.1 \text{ nmol/h per mg}$ of protein (means $\pm$ S.D. for three experiments).

Fig. 4. Concentration-dependence of the incorporation of phosphatidyl[^{14}C]glycerol into the lipoteichoic acid-containing fraction

The indicated amounts of phosphatidyl[^{14}C]glycerol were incubated with 750 μg of particulate fraction in a total volume of 125 μl of assay buffer. Other details are described under 'Experimental'. Inset shows a Lineweaver-Burk plot. Values are means for three experiments with a variation coefficient of less than 7%. Incorporation under standard conditions (i.e. at 180 nmol of phosphatidylglycerol) was $1.63 \pm 0.11 \text{ nmol of phosphatidylglycerol/h per mg of protein}$.

Role of other precursors in the biosynthesis of lipoteichoic acid

Lipoteichoic acids of *B. bifidum* subsp. *pennsylvanicum* contain high amounts of glucose or galactose present in a polysaccharide end chain (Op den Camp *et al.*, 1984). In order to determine the biosynthetic origin of this chain we measured the incorporation of UDP-[^{14}C]glucose or UDP-[^{14}C]galactose, in the presence of unlabelled UDP-galactose or UDP-glucose, into lipoteichoic acid by using the method described for phosphatidylglycerol. Products were analysed by Sepharose 6B column chromatography. We also used the glycosyltransferase assay described by Arakawa *et al.* (1981) to study the incorporation of the labelled UDP-glycosides. In this method purified lipoteichoic acid was used as acceptor molecule. Products were characterized by paper chromatography (Arakawa *et al.*, 1981) or Sepharose column chromatography. In both assays we could not detect high M_r products, although we varied incubation time, pH, specific activity of precursors, Mg^{2+} concentration and buffer composition. Since the biosynthetic activity of the membrane preparation was rather low, we cannot exclude the nucleotide sugars as biosynthetic precursors.

To test their acceptor function, [^{14}C]oleic acid-labelled phosphogalactolipids (100 000 d.p.m./incubation) replaced phosphatidylglycerol as the labelled precursor. The assay mixture was incubated for 24 h at 37°C. Only a small amount of radioactivity (520 ± 180 d.p.m.) became saline-extractable during the incubation. Since this amount of radioactivity was too low to be used for definitive characterization we may only speculate about the function of the phosphogalactolipids as lipid acceptors for the polymerization of the poly(glycerol phosphate) backbone.

Discussion

The synthesis of the bifidobacterial lipoteichoic acid by transfer of *sn*-glycerol 1-phosphate units from phosphatidylglycerol is in agreement with its poly(1,2)glycerolphosphate structure (Op den Camp *et al.*, 1984). Lipid 6, the presumable lipid anchor (Op den Camp *et al.*, 1984) most probably serves as point of initiation, although this could not definitely be proved. The results are comparable with those described for synthesis of the poly(1,3)-glycerolphosphate backbone in *Staphylococcus aureus* (Glaser & Lindsay, 1974; Koch *et al.*, 1984), *Lactobacillus casei* (Taron *et al.*, 1983) and streptococci (Emdur & Chiu, 1974, 1975; Ganfield & Pieringer, 1980). CDP-glycerol, precursor of wall teichoic acid synthesis (Ward, 1981), could be excluded as precursor of the poly(glycerol phosphate) backbone, since CDP-glycerol did not inhibit the incorporation of phosphatidylglycerol and CDP-[^{14}C]glycerol was not incorporated into lipoteichoic acid. This is in agreement with our understanding of the structure of the lipoteichoic acids of *B. bifidum* subsp. *pennsylvanicum*, since a poly(1,2)glycerolphosphate chain cannot be synthesized from CDP-glycerol, transferring a *sn*-glycerol 3-phosphate group.

The absence of an effect of Mg^{2+} on the incorporation was also reported by Ganfield & Pieringer (1980). In contrast with these findings Emdur & Chiu (1975) found a stimulating effect of Mg^{2+} ions. Previously (Veerkamp, 1976), we observed that the synthesis of diphosphatidylglycerol from phosphatidylglycerol was stimulated by Mg^{2+} . Therefore it may be expected that Mg^{2+} ions play an important role in the regulation of the phosphatidylglycerol turnover.

Phosphatidylglycerol has been described as a precursor for several membrane components in Gram-positive bacteria (Koch *et al.*, 1984; Koga *et al.*, 1984). For *B. bifidum* subsp. *pennsylvanicum* we have now established the role of this molecule in the synthesis of diphosphatidylglycerol, the phosphogalactolipids (Veerkamp, 1976; Van Schaik & Veerkamp 1975, 1976) and lipoteichoic acid.

Pulse-chase experiments *in vivo* would give more information about the rate of the several reactions, but these are obviated by the fact that *B. bifidum* subsp. *pennsylvanicum* cannot incorporate glycerol due to the absence of glycerol kinase (Molenkamp & Veerkamp, 1976).

Normally, glycosylation of glycerol units of lipoteichoic acids occurs with UDP-glycosides as precursors (Arakawa *et al.*, 1981; Emdur & Chiu, 1975; Mancuso *et al.*, 1979). The lipoteichoic acid of *B. bifidum* subsp. *pennsylvanicum* consists, however, of an unsubstituted backbone with a glucose or galactose polymer linked to the terminal glycerol (Op den Camp *et al.*, 1984). UDP-glucose and UDP-galactose were not incorporated into the lipoteichoic acid by the particulate membrane fraction of *B. bifidum*. This may be due to the low biosynthetic activity of the fraction or the polysaccharide part may be synthesized on a lipid intermediate and then transferred to the lipoteichoic acid. The use of such a lipid for glycosylation was recently described in *Streptococcus sanguis* (Mancuso & Chiu, 1982). The biosynthetic reactions leading to the synthesis and transfer of the polysaccharide part remain to be established.

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