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Mode of Elongation of the Glycerol Phosphate Polymer of Membrane Lipoteichoic Acid of *Streptococcus faecium* ATCC 9790

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Specific degradation of membrane lipoteichoic acid of *Streptococcus faecium* ATCC 9790 by a phosphodiesterase from *Aspergillus niger* and by periodate oxidation has demonstrated that the enzymatic synthesis of the glycerol phosphate polymer of the molecule occurs by an external elongation system. Evidence of this type of mechanism was obtained with lipoteichoic acid synthesized in vivo or in vitro by differential radioisotope labeling techniques. The glycerol phosphate repeating units were transferred from phosphatidylglycerol and became linked through a phosphodiester bond to the glycerol phosphate unit of the chain farthest from or most external to the lipid end of the polymer.

The biosynthesis of the polymer portion of bacterial lipopolysaccharides (the O-antigen region) and peptidoglycans has been shown to occur by a chain elongation mechanism that is quite different from that involved in the synthesis of wall teichoic acids (7). Chain extension in the former case is brought about by the transfer of the extending chain from a carrier lipid to a single repeating unit also bonded to a carrier lipid. The net effect of this mechanism places the newly added repeating unit at an internal position between the growing chain and the lipid moiety. In wall teichoic acid synthesis, the chain elongation reaction places the newly added repeating unit at a terminal, external position which is farthest from the lipid end of the polymer (7-9). The mechanism of extension of the polyglycerol phosphate chain of membrane lipoteichoic acid has not been established. Although the structure of the polyglycerol phosphate chain of certain wall teichoic acids appears to be similar to the glycerol phosphate polymer of membrane lipoteichoic acid, the manner by which the chain is elongated need not be the same. The latter is particularly true since the donor of the glycerol phosphate units of wall teichoic acids appears to be CDP-glycerol (9), whereas the glycerol phosphate moieties of membrane lipoteichoic acids are derived from phosphatidylglycerol (3, 5, 6). The lipophilic properties of phosphatidylglycerol, which functions in the hydrophobic environment of the membrane-bound enzyme, could alter the mode of chain elongation from an external to an internal mechanism.

In an effort to clarify this problem we studied the in vitro and in vivo biosynthesis of the membrane lipoteichoic acid of *Streptococcus faecium* ATCC 9790 by differential radioisotope labeling techniques. The results support an external elongation mechanism for the biosynthesis of the glycerol phosphate polymer of membrane lipoteichoic acids.

MATERIALS AND METHODS

Preparation of lipoteichoic acid-synthesizing enzyme. *Streptococcus faecium* ATCC 9790 was grown to mid-log phase in a chemically defined medium (13, 14). Protoplasts prepared by the method of Roth et al. (10) were osmotically lysed in 100 ml of 0.05 M Tris-hydrochloride (pH 7.5)-0.01 M $MgCl_2$-0.001 M EDTA containing about 2 mg each of DNase and RNase. The pelleted material obtained after centrifugation at 30,000 × *g* for 40 min was resuspended in 10 ml of the above buffer and centrifuged at 1,000 × *g* for 2 min to remove unbroken cells. The 1,000 × *g* supernatant was then lyophilized and stored as a dry powder at −20°C until used as the source of the membrane-bound enzyme. All manipulations were carried out at 4°C.

In vitro synthesis of lipoteichoic acid. Membrane lipoteichoic acid was enzymatically synthesized in vitro by the method of Ganfield and Pieringer (5). Membrane [1(3)-*glycerol*-^{14}C] lipoteichoic acid (100,800 cpm per tube) from *Lactobacillus fermentum* NCTC 6991 and [1(3)-*glycerol*-^{3}H]-phosphatidylglycerol (279,600 cpm per tube) were used in various combinations as the radioactive substrates in a reaction mixture containing Triton X-100 (0.2%), 2-mercaptoethanol (12 mM), Tris-hydrochloride buffer (pH 8.0, 24 mM), and 400 μg of protein of the membrane-bound enzyme from *S. faecium* in a final volume of 125 μl. It should be noted that the enzyme preparation also contains endogenous nonradioactive phosphatidylglycerol and lipoteichoic acid (5). Controls in which

enzyme is added immediately before stopping the reaction were incubated under the same conditions of temperature (37°C) and time (7 h) as were the enzyme-containing tubes. The reaction was stopped with 2 ml of $CHCl_3$:CH_3OH (1:1, vol/vol), and the denatured protein was collected as a pellet after centrifugation at 5,000 × *g* for 10 min. The pellet was washed with 1 ml of $CHCl_3$ and then extracted with 1 ml of 0.2% NaCl. The radioactivity in the extract was determined in a Tracor scintillation spectrometer adjusted to discriminate between ^3H and ^{14}C.

Preparation and assay of glycerophosphodiesterase and phosphomonoesterase from *Aspergillus niger*. A glycerophosphodiesterase preparation, partially purified form *A. niger* by the method of Schneider and Kennedy (11), was generously given to us by Eugene P. Kennedy. This preparation was free from phosphomonoesterase activity. Fischer et al. (4) found that if the crude extract from *A. niger* was not purified as much as the Schneider and Kennedy preparation, both a phosphomonoesterase as well as a phosphodiesterase activity could be detected. The latter enzymes were extracted from 5 g of Rhozyme HP150 (Rohm and Haas Co.) with 200 ml of 50 mM sodium acetate (pH 4.0) and subsequently precipitated by the addition of 30 and 55% acetone (11). The acetone (30 to 55%) particulate fraction was redissolved in 50 mM ammonium acetate (pH 4.0, 20 ml) and reprecipitated with ammonium sulfate (final concentration, 3.2 mM) at 4°C. This step was repeated several times. The final particulate preparation was dissolved in 50 mM ammonium acetate, pH 4.0, (10 ml) and stored at 4°C (4). The enzymes were assayed after purification using [1(3)-*glycerol*-^{14}C]-glycerophosphorylglycerophosphorylglycerol (obtained by deacylating diphosphatidylglycerol) as the substrate in 0.1 M ammonium acetate (pH 4.0) incubated at 37°C for up to 3.5 h. The reaction was stopped by adding a drop of concentrated ammonium hydroxide. [^{14}C]Glycerol released during the enzymatic reaction was isolated by chromatography on Whatman no. 1 paper developed with 1 M NH_4Ac (pH 7.5)-95% ethanol (30:70, vol/vol) (system A) or on Whatman DEAE paper developed with water (system B).

Preparation of radioactive substrates. [1(3)-*glycerol*-^{14}C]lipoteichoic acid was isolated from *Lactobacillus fermentum*. *L. fermentum* NCTC 6991 (a gift from the Department of Microbiology and Immunology, Temple University School of Medicine) was grown in 10 ml of a chemically defined medium (2) containing 30 μCi of [1(3)-^{14}C]glycerol (51.3 mCi/mmol) to late log phase. The harvested cells were extracted sequentially with $CHCl_3$:CH_3OH (2:1, vol/vol) and phenol-water (1:1, vol/vol) at 65°C and then dialyzed for several days at 4°C against many changes of water. The nondialyzable water-soluble fraction was extracted with $CHCl_3$. All radioactivity in this fraction could be recovered in the excluded volume of a G-25 Sephadex column ([^{14}C]glycerol is not excluded from this molecular sieve). [1(3)-*glycerol*-^3H]phosphatidylglycerol was prepared by growing *Pseudomonas diminuta* in 500 ml of media containing 1 mCi of [1(3)-^3H]glycerol (2.5 Ci/mmol) under conditions previously carried out in our laboratory (12). The glycerol moieties of phosphatidylglycerol are labeled equally (12).

RESULTS AND DISCUSSION

Preparation of membrane lipoteichoic acid of *S. faecium* growth at different stages in the presence of radioactive glycerol. *S. faecium* was grown in the presence of [1(3)-^{14}C]glycerol in a chemically defined medium (13) for three different periods of time. In the first set of conditions, the *S. faecium* was exposed to 10 μCi of [1(3)-^{14}C]glycerol in 10 ml of medium from the time of inoculation (absorbancy at 675 nm [A_{675}], 0.065) to late log phase (A_{675}, 0.94). In set 2, the cells were grown from A_{675} of 0.065 to A_{675} of 0.55 with [^{14}C]glycerol present and then in the absence ([^{14}C]glycerol was removed by washing the cells several times with fresh medium) of radioactive glycerol until an A_{675} of 1.1 was reached. In set 3, the conditions were reversed. The cells were grown in the absence of [1(3)-^{14}C]glycerol from A_{675} of 0.065 to A_{675} of 0.8, which point 10 μCi of [1(3)-^{14}C]glycerol was added, and growth was allowed to proceed to an A_{675} of 0.95. The cells from each of the sets were collected and washed with water on 0.4-μm GF/C filters (Whatman). The lipids were extracted from the cells with 7.6 ml of $CHCl_3$:CH_3OH:H_2O (1:2:0.8, vol/vol) (1). The addition of 2 ml of $CHCl_3$ and 2 ml of H_2O yielded a two-phase system. The lower $CHCl_3$ phase was removed, and the upper aqueous phase was back extracted several times with $CHCl_3$. The denatured protein and the aqueous phase were heated in a boiling water bath for 3 h. Particulate matter was removed by filtration through a-0.4 μm GF/C filter. The filtrate was adjusted to 5 ml, and the lipoteichoic acid was analyzed by phosphodiesterase and periodate oxidation techniques. A similar water extraction procedure has been used to isolate lipoteichoic acid from *L. fermentum* NCTC 6991 (16). Because the hot water technique extracts membrane lipoteichoic acid (16) and since no glycerol-containing polymer has been isolated from the wall of *S. faecium* (G. D. Shockman, personal communication, and W. Fischer, personal communication), the [1(3)-*glycerol*-^{14}C]-containing polymer (size indicated by its exclusion from Sepharose 6B) was considered to be the membrane lipoteichoic acid of *S. faecium*.

Removal of the terminal glycerol from the lipoteichoic acid by treatment with glycerophosphodiesterase. The specificity of glycerophosphodiesterase from *A. niger* purified as directed by Schneider and Kennedy (11) should cause the release of only the terminal glycerol from the polyglycerol phosphate chain of the membrane lipoteichoic acid. The amount of terminal [^{14}C]glycerol released compared with the total [^{14}C]glycerol in the polymer synthesized in vivo under the three sets of conditions

given above should indicate if an internal or external chain elongation system is functioning. The [^{14}C]lipoteichoic acid together with glycerophosphodiesterase (1 mg) was dissolved in 0.15 ml of 0.1 M ammonium acetate, pH 4.0, and allowed to react for 3 h at 37°C. A 25-μl aliquot from each set was chromatographed in system B. The anionic lipoteichoic acid remained at the origin, and the nonionic glycerol traveled to the solvent front. The radioactivity in the two compounds was determined by counting the appropriate areas of the chromatogram in a scintillation spectrometer. Controls containing unreacted lipoteichoic acid were also chromatographed and counted. Small amounts of [^{14}C]-glycerol in the lipoteichoic acid preparations were subtracted from the total [^{14}C]glycerol found in the phosphodiesterase-containing tubes. The entire procedure beginning with the growth of *S. faecium* under the three sets of conditions was carried out in duplicate. The percentage of [^{14}C]glycerol released from the terminal position farthest from the lipid end of the polymer is shown in Table 1. The membrane lipoteichoic acid containing the highest percentage of its radioactive glycerol in the terminal exposed position (capable of being acted upon by the phosphodiesterase) is that of set 3 (Table 1) in which [^{14}C]glycerol would have been added last to the nonradioactive glycerol polymer. The least [^{14}C]glycerol released from the terminal position is in set 2 (Table 1), in which nonradioactive glycerol would have been added to radioactive polyglycerolphosphate. An intermediate percentage is liberated from the lipoteichoic acid of *S. faecium* grown continuously in the presence of [^{14}C]glycerol (set 1). These data favor an external addition mechanism.

TABLE 1. *Analysis of differentially labeled lipoteichoic acid of S. faecium with glycerophosphodiesterase of A. niger*[a]

Set	Growth (A_{675}) of *S. faecium* in presence (+) and absence (−) of [^{14}C]glycerol	%[^{14}C]Glycerol released from [^{14}C]lipoteichoic acid by phosphodiesterase in 3 h
1	(+) 0.065 → 0.95	2.89 (4.55)
2	(+) 0.065 → 0.55; (−) → 1.1	0.57 (0.67)
3	(−) 0.065 → 0.8; (+) → 0.95	10.48 (10.20)

[a] *S. faecium* was grown in duplicate in the presence and absence of 10 μCi [1(3)-^{14}C]glycerol at different stages of growth (turbidity was measured at 675 nm). Lipoteichoic acid extracted from the cells was reacted with the glycerophosphodiesterase of *A. niger*. Duplicate values are in parentheses. The amounts of [^{14}C]lipoteichoic acid incubated with enzyme were set 1, 43,660 cpm (52,040 cpm); set 2, 77,380 cpm (67,500 cpm); and set 3, 19,000 cpm (19,300 cpm).

Periodate oxidation of the terminal glycerol of membrane lipoteichoic acid synthesized in vivo. The terminal glycerol is linked to the polymer through a phosphodiester bond at a primary alcohol function (5, 15) and should be the only glycerol unit of the chain capable of releasing formaldehyde upon exposure to periodate. The specificity of this reaction can be used to confirm the results of the phosphodiesterase experiments. Membrane lipoteichoic acid was obtained from *S. faecium* grown in duplicate under the same sets of conditions described above. The [1(3)-*glycerol*-^{14}C]lipoteichoic acid was reacted with 0.5 ml of periodic acid (8.8 μmol per 50 ml) in the presence of 3 ml of 0.1 M phosphate buffer (pH 7.2) 25 μl of formaldehyde, and 1.5 ml of water for 1 h in the dark at room temperature. The reaction was stopped by the addition of 0.5 ml of glucose (1 μmol per 0.5 ml). Twenty-five milliliters of 0.4% dimedon was added and kept at 4°C overnight. The quantitatively precipitated formaldehyde-dimedon derivative was collected on filters, dried, and weighed. The residue was dissolved in ethanol, and a sample was counted in a scintillation spectrometer. The dimedon derivative was recrystallized four times until a constant specific activity was attained. Since formaldehyde-dimedon represents one-half of the potential radioactivity in the terminal [1(3)-^{14}C]glycerol, the radioactivity in the formaldehyde was multiplied by two to calculate the percent of radioactivity in the terminal glycerol (Table 2). In confirmation of the phosphodiesterase experiment, periodate oxidation released more radioactive formaldehyde from the lipoteichoic acid derived from cells

TABLE 2. *Analysis of differentially labeled lipoteichoic acid of S. faecium by periodate oxidation*[a]

Set	Growth (A_{675}) of *S. faecium* in presence (+) and absence (−) of [^{14}C]glycerol	%[^{14}C]Formaldehyde (×2) released by IO_4 in 1 h from [^{14}C]-lipoteichoic acid
1	(+) 0.065 → 0.88	9.04 (9.04)
2	(+) 0.065 → 0.58; (−) → 1.0	3.96 (6.60)
3	(−) 0.065 → 0.58; (+) → 0.9	14.87 (19.82)

[a] *S. faecium* was grown in duplicate in the presence and absence of 10 μCi [1,3-^{14}C]glycerol at different stages of growth (turbidity was measured at 675 nm). Lipoteichoic acid extracted from the cells was reacted with periodic acid for 1 h in the dark at room temperature. Released formaldehyde was trapped as the dimedon derivative and recrystallized to constant specific activity. Duplicate values are in parentheses. The amounts of [^{14}C]lipoteichoic acid reacted with periodic acid were set 1, 69,530 cpm (109,190 cpm); set 2, 48,950 cpm (72,170 cpm); and set 3, 6,720 cpm (9,540 cpm).

grown first in the absence and then in the presence of [^{14}C]glycerol (set 3, Table 2) and released less radioactive formaldehyde when cells were grown first in the presence and then in the absence of [^{14}C]glycerol (set 2, Table 2). Therefore, we conclude that the glycerol phosphate repeating unit is added to the external glycerol of the polymer.

Order of addition of glycerol phosphate from phosphatidylglycerol to lipoteichoic acid in vitro. The biosynthesis of membrane lipoteichoic acid in vitro is perhaps a better defined system than its formation in vivo since the product is formed directly from two substrates rather than from an indirect precursor, such as glycerol, which may take a rather circuitous, multistep pathway before becoming incorporated into the product. The mechanism of elongation of the glycerol phosphate polymer of lipoteichoic acid formed in vitro by the sequential addition of glycerol phosphate units from phosphatidylglycerol was tested by using double isotope labeling techniques. Tritium-labeled glycerol phosphate from [1(3)-*glycerol*-^{3}H]phosphatidylglycerol (^{3}H-PG) was incorporated into [1(3)-*glycerol*-^{14}C]lipoteichoic acid [LTA-(^{14}C-GP)] (from *L. fermentum*) when incubated in the presence of a membrane-bound enzyme (from broken cells of *S. faecium*) under conditions described above. The following reaction would appear to be occurring:

$$\text{LTA-}(^{14}\text{C-GP})_x + (^{3}\text{H-PG})_n \rightarrow$$

$$\text{LTA}(^{14}\text{C-GP})_x\text{-}(^{3}\text{H-GP})_n + (^{3}\text{H-diacylglycerol})_n$$

The location of ^{14}C- and ^{3}H-labeled glycerol in the polymer was determined with the aid of an enzyme preparation from *A. niger* which contained both phosphodiesterase and phosphomonoesterase activities. This preparation would be expected to catalyze the sequential hydrolysis of glycerol and phosphate groups from the polymer beginning at the nonlipid end of the chain. When a sample of [1(3)-glycerol-^{14}C]lipoteichoic acid from a control tube (*S. faecium* enzyme added after a 7-h incubation and immediately denatured) was treated with the crude *A. niger* phosphomonoesterase and diesterase preparation for 4 h (see above for conditions of assay), 67.8% (1,786 cpm of [^{14}C]glycerol/2,633 cpm of total ^{14}C × 100) of the total ^{14}C label was released as free glycerol (identified by chromatography in system A). Treatment of a portion of the [*glycerol*-^{14}C,^{3}H]lipoteichoic acid (synthesized from an incubation containing all reagents and *S. faecium* enzyme) with the *A. niger* preparation for 4 h released 39.1% (3,034 cpm of [^{14}C]glycerol/7,865 cpm of total ^{14}C × 100) free glycerol. These results suggest that a significant portion of the nonlipid, outermost end of the [^{14}C]glycerol phosphate polymer was covered with [^{3}H]glycerol phosphate from the added [^{3}H]phosphatidylglycerol or with nonradioactive glycerol phosphate derived from endogenous nonradioactive phosphatidylglycerol in the enzyme preparation. Examination of the amount of [^{3}H]glycerol versus [^{14}C]glycerol released by the *A. niger* hydrolytic enzymes supports this conclusion. One-hour exposure to these phosphoesterase enzymes removes 60.2% (5,474 cpm of [^{3}H]glycerol/9,090 cpm total ^{3}H × 100) of the ^{3}H label as free glycerol, whereas only 2.6% (210 cpm of [^{14}C]glycerol/8,290 cpm of total ^{14}C × 100) of the original ^{14}C label in the lipoteichoic acid is freed as [^{14}C]glycerol. These data are consistent with the view that membrane lipoteichoic acid is synthesized by a mechanism in which the repeating glycerol phosphate units are added from phosphatidylglycerol to the external position of the elongating chain.

Since the enzyme catalyzing the formation of the glycerol phosphate polymer and the donor of the glycerol phosphate, phosphatidylglycerol, are membrane bound (5), an external elongation mechanism would require the terminal glycerol of the chain to be in close proximity of the membrane during the transfer of the next glycerol phosphate unit to the polymer. Although it is easy to understand how the lipid end of the molecule would anchor the polymer to the hydrophobic membrane, it is not as readily apparent how the nonlipid end of the polymer maintains contact with the membrane-bound enzyme. The elongating chain with its increasing hydrophilic forces must either remain in the membrane or bend back in a loop to the membrane to interact with the transferase and bring about the addition of the next glycerol phosphate unit. It is perhaps this constraint that terminates the elongation process and limits the length of the polymer.

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