The Synthesis of Polyribitol Phosphate

II. ON THE MECHANISM OF POLYRIBITOL PHOSPHATE POLYMERASE*

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SUMMARY

The synthesis of polyribitol phosphate by polyribitol phosphate polymerase from Staphylococcus aureus H proceeds by a single chain mechanism so that the enzyme completes a chain of approximately 30 units linked to lipoteichoic acid carrier, before starting a new chain. The lipoteichoic acid carrier of Staphylococcus aureus and Bacillus subtilis are interchangeable and can be used both for the synthesis of polyglycerol phosphate or polyribitol phosphate. Both of these polymers appear to be attached to the same site on the polyglycerolphosphate or polyribitol phosphate. No evidence has been found for the involvement of an additional carrier such as undecaprenol phosphate in polyribitol phosphate synthesis.

In the preceding paper we described the preparation of polyribitol phosphate polymerase from Staphylococcus aureus H and an acceptor designated as lipoteichoic acid carrier (1). In this communication, we examine the mechanism of the reaction and consider first the regulation of polyribitol phosphate chain length and, second, the distribution and species specificity of LTC, and finally the possible involvement of additional intermediates in polyribitol phosphate synthesis.

Materials and Methods

Enzymes and substrates were the same as those used in the preceding paper (1). Lysostaphin was obtained from Schwarz-Mann. Polyglycerolphosphate polymerase and Bacillus subtilis LTC labeled with [3H]glycerol were prepared as described by Mauck and Glaser (2). The following media were used for the growth of bacterial strains listed in Table I below: Bacillus subtilis, Bacillus licheniformis, Staphylococcus aureus, Corynebacterium xerosis, Micrococcus lysodeikiticus, Arthrobacter globiformis and Mycobacterium smegmatis were grown in antibiotic Medium 3 (Difco); L. plantarum and Lactobacillus casei and Staphylococcus faecalis were grown without aeration in Laetococcus M18S broth (Difco); Escherichia coli B was obtained from Grain Processing Corporation, and Mycobacterium phlei was a gift of Dr. R. Fall of this Department. Streptococcus pneumoniae was grown in Brain Heart Infusion Broth (Difco) without aeration. Cells were harvested in logarithmic phase of growth, broken with glass beads and LTC extracted from the membranes with Triton as described for S. aureus (1) and also by the phenol procedure (3). Both extracts were tested for LTC activity with identical results.

RESULTS AND DISCUSSION

Single Chain Mechanism of Polyribitol Phosphate Synthesis—When polyribitol phosphate synthesis is examined as a function of time (Fig. 1) by polyacrylamide gel electrophoresis, the polyribitol phosphate appears as a single sharp band linked to acceptor. No intermediate sizes are seen, and LTC appears either as fully loaded LTC or as free LTC. Our interpretation of these observations is that the enzyme completes a polyribitol phosphate chain before starting a new chain, and that chain length is finite. If additional LTC is added after 3 hours (Fig. 2), it becomes fully loaded showing that the enzyme is not inhibited by loaded LTC.

The data in Fig. 3 show that if fully loaded LTC is incubated for 24 hours with additional enzyme and CDP-ribitol, there is only a small increase in chain length. It should be noted that as shown in the preceding paper, the enzyme is stable under assay conditions for 24 hours (1).

The chain length of polyribitol phosphate was determined as a sample identical with that of Fig. 2B. Loaded LTC was separated from residual CDP-ribitol by chromatography on Bio-Gel P-30 in 0.05 M Tris-HCl-0.001 M 2-mercaptoethanol-0.1% Triton, pH 8.0. An aliquot of the polymer containing 94,500 cpm was oxidized with periodate and the formaldehyde dimedon complex isolated with carrier as described previously (1). Five-hundred seventy counts per minute were found in formaldehyde; therefore the chain length was 30 units, in reasonably good agreement with the value found in other strains of S. aureus for intact cell wall polyribitol phosphate (4).

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of ribitol-P per μmole of LTC phosphate. Since the chain length of polyribitol phosphate is 30 units, the maximum size of LTC is 12 glycerol units. Thus, LTC is a rather small molecule with a molecular weight approximately 2,800 as the sodium salt and, when fully loaded with polyribitol phosphate, has a molecular weight of approximately 11,000 calculated as the sodium salt. The fact that these small molecules are retained by an Amicon XM-50 membrane probably reflects both aggregation and the rigid shape of these highly charged molecules. Indeed, at high ionic strength (greater than 0.3 M NaCl) a considerable fraction of S. aureus LTC passes through an XM-50 membrane.

Based on the availability of membrane teichoic acid to external antibody, Knox and Wicken (5) have suggested that the lipoteichoic acid may traverse the cell wall. The chain length of the S. aureus LTC is too short for it to traverse the cell wall and be exposed on the cell surface.

The synthesis of a polymer of defined length is puzzling; it
as described in the preceding paper (1), using five different fractions across the LTC peak eluted from DEAE-Bio-Gel and was found to be constant. The capacity was determined by loading limiting concentrations of LTC with CDP-[γ-32P]ribo'tol, the reaction mixtures were then subjected to polyacrylamide gel electrophoresis and the 32P content in the region of loaded LTC was determined. This cumbersome method was used because it was found that the Millipore assay yielded low values for ribitol-P incorporation compared to acrylamide gels. This difference which is about 2 to 2.5 times is presumably due to the fact that polyribitol-P is not all on the surface of the Millipore filter, thus decreasing the apparent 14C content.

implies that when this chain length is reached, binding to the enzyme is decreased. We speculate that enzyme activity occurs in a hydrophobic complex of LTC, phospholipid and enzyme, and that when a long chain of polyribitol phosphate has become attached to LTC, it becomes much more hydrophilic and dissociates from the complex. Experiments are in progress to
determine whether such a complex exists and whether loaded LTC dissociates from it. 

**Strain Specificity of LTC**—In the preceding paper we showed that polyribitol phosphate polymerase from *S. aureus* cannot use CDP-glycerol as a substrate (1), and similarly CDP-ribitol is not a substrate for the polyglycerolphosphate polymerase from *B. subtilis* (3). The LTC obtained from both of these organisms is a polyglycerolphosphate derivative and it was of interest to determine the specificity of these enzymes for LTC. In Fig. 4 we show that *B. subtilis* LTC will fully substitute for *S. aureus* LTC, for the synthesis of polyribitol phosphate. The maximal velocity and the concentration of polymer required for half-maximal velocity were essentially the same whether LTC from *B. subtilis* or LTC for *S. aureus* were used. That this LTC is acting as an acceptor is shown in Fig. 5 where the size of [PH]-glycerol-labeled *B. subtilis* LTC is changed when incubated with CDP-ribitol. It should be noted that all of the *B. subtilis* LTC can be loaded with polyribitol phosphate; thus the activity of *B. subtilis* LTC is not due to a minor contaminant present in the preparation. *B. subtilis* (ATCC 6051) from which the LTC was obtained does not contain polyribitol phosphate in the cell wall (6).

The converse experiment is shown in Fig. 6 where we show that *S. aureus* LTC acts as an acceptor for the *B. subtilis* polyglycerolphosphate polymerase. The difference in size between LTC-polyribitol phosphate and LTC-polyglycerolphosphate is independent of the source of LTC and therefore only a function of the specificity of the polymerase.

Because LTC from *B. subtilis* and *S. aureus* appear to be interchangeable, we have examined a broad distribution of bacterial species for the presence of LTC by extracting a crude preparation of cell wall material and assaying for the presence of LTC. The results of these experiments have been summarized in Table I. The data show that LTC is present in all of the species examined, and that the LTC from these organisms behaves as a polyribitol phosphate polymerase.

**Fig. 3.** Effect of time of incubation on the size of polyribitol phosphate. A, [32P]LTC from *Staphylococcus aureus* was loaded with polyribitol for 3 hours under conditions of Fig. 1 and chromatographed on acrylamide gels as shown in Fig. 1. B, to a second sample fresh CDP-ribitol and additional enzyme were added after 3 hours and incubation continued for 24 hours. Polyribitol phosphate polymerase remains active for 24 hours under assay conditions (1).

**Fig. 4.** Dependence of polyribitol phosphate synthesis on *Bacillus subtilis* LTC. Standard assay conditions were used with the indicated concentrations of *B. subtilis* LTC (expressed as nanomoles of phosphorus).

**Fig. 5.** Synthesis of polyribitol phosphate with *Bacillus subtilis* LTC. [2-3H]-glycerol-labeled *B. subtilis* LTC (2) was incubated under standard assay conditions with 6.5 units of polyribitol phosphate polymerase for 24 hours at 37°C. From left to right the panels show: (a) the original *Bacillus subtilis* LTC; (b) a mixture of loaded and unloaded LTC; and (c) fully loaded *B. subtilis* LTC. Nine-centimeter gels were used.

**Fig. 6.** Synthesis of polyribitol phosphate with *Bacillus subtilis* LTC. [2-3H]-glycerol-labeled *B. subtilis* LTC (2) was incubated under standard assay conditions with 6.5 units of polyribitol phosphate polymerase for 24 hours at 37°C. From left to right the panels show: (a) the original *Bacillus subtilis* LTC; (b) a mixture of loaded and unloaded LTC; and (c) fully loaded *B. subtilis* LTC. Nine-centimeter gels were used.
Effect of preloading with polyribitol phosphate on LTC activity. *Staphylococcus aureus* [32P]LTC was loaded under standard assay conditions for 24 hours with 6.5 units of polyribitol phosphate polymerase and 110 nmoles of CDP-ribitol and chromatographed on 9-cm polyacrylamide gels (A). A duplicate sample after 24-hour incubation was heated at 100° for 2 min to inactivate the polymerase and then incubated with 4.5 units of *Bacillus subtilis* polyglycerolphosphate polymerase and 270 nmoles of CDP-glycerol and 1 μg of *Escherichia coli* alkaline phosphatase for 3 hours at 37° before chromatography on acrylamide gels (B). In the experiment shown in C the conditions were identical with B, but *S. aureus* ['C]LTC was added together with polyglycerolphosphate polymerase. In D, *S. aureus* [PC]LTC was loaded with polyglycerolphosphate polymerase. ○, 32P; O, 14C.

![Graph](http://www.jbc.org/)

**Fig. 6.** Synthesis of polyglycerol-phosphate with *Staphylococcus aureus* LTC. [32P]LTC was incubated with *Bacillus subtilis* polyglycerolphosphate polymerase, 1 hour at 37° and the reaction terminated at times indicated by the addition of sodium deoxycholate and heating to 100°, followed by chromatography on 9-cm polyacrylamide gels. The reaction mixture contained (100 μ1): 0.05 M Tris-C1, pH 8.0, 0.06 M MgCl2, 0.1% Triton, 270 nmoles of CDP-glycerol, 4.5 units of polyglycerolphosphate polymerase added initially and an additional 1.3 units after 1-hour incubation, and 1 μg of *Escherichia coli* alkaline phosphatase to destroy CMP formed during the reaction. For comparison the position of unloaded LTC and LTC fully loaded (24-hour incubation) with *S. aureus* polyribitol phosphate polymerase are also shown. The arrows indicate the position of LTC fully loaded by the polyribitol phosphate polymerase.

**Table I**

| Bacteria                  | ATCC No. | Teichoic acid in cell wall | LTC activity | Reference |
|---------------------------|----------|---------------------------|--------------|-----------|
| *Staphylococcus aureus* H | 6061     | R                         | +            | (4)       |
| *Bacillus subtilis*       | 9045     | G                         | +            | (6)       |
| *Bacillus licheniformis*  | 8014     | R                         | +            | (8)       |
| *Lactobacillus plantarum* | 7409     | R                         | +            | (9, 10)   |
| *Lactococcus casei*       | 9730     | R                         | +            | (10, 11)  |
| *Streptococcus faecalis*  | 354      | R                         | +            | (10)      |
| *Escherichia coli* B      |          |                           |              | (11)      |
| *Micrococcus lysodeikticus*| 4668     |                           |              | (12)      |
| *Mycobacterium smegmatis* | 14465    |                           |              |           |
| *Arthrobacter globiformis*| 6150     |                           |              |           |
| *Corynebacterium xerosis* | 373      |                           |              |           |
| *Streptococcus pneumonia* | 12213    |                           |              |           |

- R = polyribitol phosphate, G = polyglycerolphosphate.
- This organism in addition contains (polyglucosylglycerol-P) and polygalactosyglycerol-P.
- No glycerol or ribitol is found in cell wall hydrolysates of these strains (F. Fiedler, unpublished observation).
- This organism contains a complex polysaccharide containing ribitol phosphate in the wall.

Organisms which are known to have polyglycerolphosphate or polyribitol phosphate in the cell wall, but also in an organism such as *L. casei* in which these wall polymers are absent. The presence of LTC correlates well with the presence of a membrane teichoic acid and with antigenic studies of McCarthy in which a number of the same organisms were examined for the presence of polyglycerolphosphate (5, 14).

With LTC from *B. licheniformis*, *L. plantarum*, and *S. faecalis*, we have examined the behavior of the LTC-polyribitol phosphate formed on polyacrylamide gels, and found it identical with that formed with *S. aureus* LTC.

The data in this section clearly show that LTC is a common carrier present in many teichoic acid-synthesizing organisms,
TABLE II
Exchange between CMP and CDP-ribitol

The reaction mixture contained 2.5 μmoles of Tris-HCl, pH 8.0, 0.2 μmoles of spermidine, 0.1% Triton X-100, 10 units of Streptococcus aureus polypolyribitol phosphate polymerase, 27 μmoles of CDP[^14C]-ribitol (450 dpm per μmole), and 100 μmoles of HIP-CMP, 2.1 × 10^6 dpm per μmole and where indicated a saturating level of LTC, and was incubated at 25°C for 1 hour. The control was not incubated and contained both enzyme and LTC. CDP-ribitol was isolated from the reaction mixture by successive chromatography on Whatman No.3MM paper in (a) iso- butyric acid-NH$_4$OH (10⁻⁴ M EDTA (66:1:33); (b) ethanol-1 M NH$_4$ acetate (7.5:3); pH 3.8; (c) ethanol-1 M NH$_4$ acetate, pH 7.8 (7.5:3).

![Figure 8](http://www.jbc.org/)

FIG. 8. Polyribitol phosphate polymerase in bacitracin-inhibited cells. Staphylococcus aureus H was grown at 37°C in 600 ml of antibiotic Medium 3. At time indicated by A one flask was harvested and to another flask were added 6 ml of a solution containing 15 mg per ml of bacitracin. This second flask was harvested at Point B by centrifugation. The cells were washed with 0.05 M Tris-Cl-0.02 M spermidine-0.001 M 2-mercaptoethanol suspended in 12 ml of the same buffer and lysed at 25°C with 1.5 mg of lysozyme and 0.1 mg of RNase and DNase. Lysis was complete in 30 min, and membranes were collected by centrifugation at 100,000 × g for 1 hour, washed with same buffer and assayed by standard procedure for polyribitol phosphate polymerase (1). An aliquot of the membranes was used for the preparation of crude LTC by the Triton extraction method (1). The capacity of LTC was determined by incubating aliquots with purified polyribitol phosphate polymerase and large excesses of CDP[^14C]-ribitol at 25°C for 24 hours. The yield of polyribitol phosphate was proportional to the quantity of LTC added. Bacitracin at a concentration of 50 μg per ml was present in all buffers and assays used for Sample B. Suitable controls showed that bacitracin had no effect on enzyme activity in vitro. •, growth curve of the culture.

**Activity**

| Addition          | CDP-ribitol | Theory for % exchange |
|-------------------|-------------|-----------------------|
|                   | Hi          | Hc                    | Hi dpm |
| Control           | 360         | 6700                  | —      |
| Enzyme            | 400         | 6700                  | 1700   |
| Enzyme + LTC      | 600         | 6700                  | 1430   |

* The quantity of ^3H in CDP-ribitol if exchange proceeded at 1% of the polymerase reaction velocity.

that these molecules are to a large extent interchangeable, and that the lack of cell wall teichoic acid in an organism such as L. casei probably represents the lack of a functional polymerase and not the lack of LTC. The presence of LTC in Mycobacterium phlei is very surprising since to the best of our knowledge teichoic acids do not occur in Mycobacteria.

**Possible Intermediate Carriers in Polyribitol Phosphate Synthesis**—In a series of papers, Baddiley and co-workers have suggested that undecaprenol phosphate is involved in teichoic acid biosynthesis (15-17). The lipid intermediates have not as yet been fully characterized and their data depend on pulse chase experiments, and pertain primarily to the synthesis of heteropolymers such as the glucosylglycerol-1' polymer of polyribitol phosphate or polyglycerolphosphate synthesis (15).

We have looked for ribitol-P-undecaprenol intermediate in two ways. We have chromatographed reaction mixtures in a variety of solvents to detect a possible lipid intermediate, or extracted reaction mixtures with chloroform-methanol, 2:1, or butanol and found no evidence for the presence of a lipid-linked ribitol phosphate.

We have tested the purified polyribitol phosphate polymerase for its ability to catalyze an exchange reaction between [HI]-CMP and CDP[^14C]-ribitol under conditions where the rate of polymer synthesis is 50% inhibited by CMP. We find no evidence for such an exchange (Table II), although such an exchange has been observed with every system in which undecaprenol-P acts as a glycosyl carrier (18, 19). Added undecaprenol-P has no effect on the velocity of the purified polyribitol phosphate polymerase.

Although the presence of a lipid intermediate is difficult to rule out totally, it appears unlikely that it is involved in either polyribitol phosphate or polyglycerolphosphate synthesis (2), but may still be involved in the synthesis of the heteropolymeric teichoic acids described by Baddiley et al. (15-17).

In an attempt to examine the involvement of undecaprenol-P in *vivo*, we have examined *S. aureus* cells inhibited with bacitracin for LTC content and polyribitol phosphate polymerase activity. Bacitracin-inhibited cells have normal activity of the polymerase and normal LTC capacity (Fig. 8). Since bacitracin inhibits the dephosphorylation of undecaprenol-pyrophosphate (20), these data confirm the in *vivo* observation that undecaprenol-P is not involved in polyribitol phosphate synthesis on the assumption that the membrane contains a single pool of undecaprenol-P.

The data in Fig. 8 are also of interest because they show that the LTC pool is not saturated under conditions where peptidoglycan synthesis is inhibited, suggesting that control of polyribitol phosphate synthesis precedes the polymerization step. Otherwise, under conditions where no synthesis of peptidoglycan takes place, LTC would be totally loaded with polyribitol phosphate.

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