AN INTERMEDIATE IN TEICHOIC ACID BIOSYNTHESIS[1]

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Received August 16,1976

Summary: In the presence of UDP-N-acetyl glucosamine and CDP-glycerol a membrane preparation from Staphylococcus aureus synthesizes a lipophylic compound, which is an intermediate in the synthesis of the linkage region between teichoic acid (polyribitol phosphate) and peptidoglycan. The synthesis of this intermediate is inhibited by Tunicamycin.

The cell wall of Staphylococcus aureus H consists of a peptidoglycan to which a teichoic acid is covalently linked. The teichoic acid is a polyribitol phosphate about 30 units long, substituted by N-acetylglucosamine residues (1). Mutants are known in which no N-acetylglucosamine is attached to the ribitol residues (2). Until recently, it was believed that the polyribitol phosphate was attached directly to the peptidoglycan, presumably by a phosphodiester bond between a terminal ribitol residue and an N-acetylmuramic acid residue in the peptidoglycan. We have recently presented enzymatic evidence for the presence of a linkage region between peptidoglycan and polyribitol phosphate consisting of N-acetylglucosamine and glycerol phosphate residues. These residues are derived from UDP-GlcNAc and CDP-glycerol, respectively (3). Independently and simultaneously, work in the laboratory of Dr. J. Baddiley has provided both chemical (4) and biosynthetic evidence (5) for the presence of glycerol-P units in the linkage regions between polyribitol phosphate and peptidoglycan. In this communication, we report that it is possible to identify an intermediate in teichoic acid biosynthesis which contains

[1]Supported by Grant GM 18405 from NIH.

the linkage region attached to an as yet unidentified lipophylic material. We also show that Tunicamycin (6-9), an antibiotic which inhibits reactions of the type UDP-GlcNAc + polyprenol phosphate → UDP(UMP) + GlcNAc-polyprenol phosphate, inhibits the synthesis of this linkage region.

MATERIALS AND METHODS

The source and preparation of nucleoside diphosphate sugars was described previously (3). In all of the experiments reported, a mutant of S. aureus H 52a2 was used (2). This mutant, kindly made available by Dr. J. Park, lacks the N-acetylglucosamine residues linked to ribitol in polyribitol phosphate. Membranes were prepared by breaking frozen cells in a Braun homogenizer (3), removing whole cells and cell walls by centrifugation at 10,000 x g at 4°, and then sedimenting membranes at 105,000 x g for 1 hour. Membranes were resuspended in a buffer containing 50 mM Tris-Cl, pH 8.0, 20 mM $MgCl_2$ and 1 mM 2-mercaptoethanol at a concentration of 10 mg protein/ml, and were stored in small aliquots in liquid nitrogen. Membrane samples were thawed only once before use. The synthesis of polyribitol phosphate by membrane preparation was assayed by the filter method described previously (10-12), except that Gelman Alpha-8 filters were used, instead of the solvinert filters which are no longer available. All reactions were carried out in 50 µl of 150 mM Tris-Cl, 20 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10 mM Spermidine, 45 mM NH_4Cl and 2.8 mM ATP.

Tunicamycin was kindly made available by Dr. G. Tamura of the University of Tokyo.

RESULTS AND DISCUSSION

In previous experiments we have shown that the incorporation of ribitol-P from CDP-ribitol into cell walls of S. aureus is greatly stimulated by the addition of CDP-glycerol and UDP-GlcNAc (3). This stimulation can be prevented by the addition of Tunicamycin (unpublished observations). In Table 1 we show that the synthesis of polymeric material from CDP-ribitol catalyzed by a membrane preparation of S. aureus 52a2 is also stimulated by the addition of CDP-glycerol and UDP-GlcNAc. This stimulation is prevented by Tunicamycin, but Tunicamycin has no effect if the membranes have been preincubated with UDP-GlcNAc. It would appear from these data that the membrane preparations can synthesize the linkage region between peptidoglycan and polyribitol phosphate (3). Since no peptidoglycan precursors are added to this system, it is likely that the teichoic acid linkage region is synthesized as a membrane bound precursor before transfer to peptidoglycan.

TABLE I. Effect of Tunicamycin on Polyribitol Phosphate Synthesis

Experiment	Additions nmoles	Polyribitol-P formed dpm
I	-	5,462
	CDP-glycerol (2.5)	8,432
	UDP-GlcNAc (200)	10,170
	CDP-glycerol (2.5) + UDP-GlcNAc (200)	16,358
	CDP-glycerol(2.5) + UDP-GlcNAc (200) + TM	9,874
II	-	10,972
	+ CDP-glycerol (2.5)	25,952
	+ CDP-glycerol (2.5) + TM	22,300

S. aureus membranes (100 µg protein) were preincubated for 15 minutes at 25°, either with no addition (Experiment I) or with 200 nmoles of UDP-GlcNAc (Experiment II). At the end of the preincubation the nucleotides listed were added as well as 25 nmoles of CDP-[^{3}H]-ribitol, 40 dpm/pmole. After incubation for 45 minutes, the amount of polyribitol phosphate formed was determined (10). Where indicated, Tunicamycin (TM) was added at a concentration of 5 µg/ml.

We have looked for potential intermediates in this process by incubating membrane preparations with UDP-[^{3}H]-GlcNAc or CDP-[^{3}H]-glycerol. With UDP-[^{3}H]-GlcNAc as a precursor, we get a very large incorporation into polymeric material. The synthesis of this polymer(s) is not inhibited by the addition of Tunicamycin, and therefore this polymeric material cannot be in intermediate in teichoic acid biosynthesis. We find no evidence that a GlcNAc polymer exists in the cell wall of S. aureus and it is unclear whether this polymer is an artifact of the in vitro system, or whether it occurs in S. aureus. S. aureus 52a2 has no

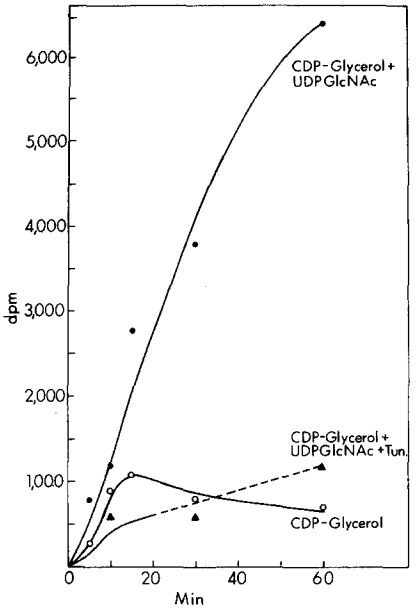

Fig. 1. Formation of polymeric material from CDP-[^{3}H]-glycerol. Standard assay conditions were used with 2.5 nmoles of CDP-[^{3}H]-glycerol (10^6 dpm). Where indicated, 200 nmoles of UDP-GlcNAc were also added. Tunicamycin was added to a concentration of 5 µg/ml. The reaction was stopped by the addition of 10 volumes of ethanol - 1 M NH_4 acetate (3/7.5) filtered and counted in a liquid scintillation counter.

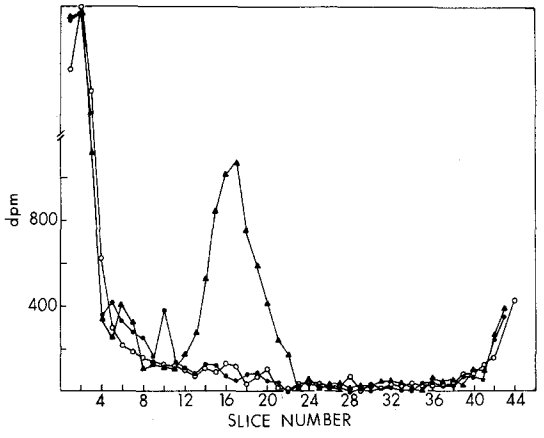

Fig. 2. SDS-Acrylamide gel electrophoresis of glycerol labelled intermediate. The reaction conditions were as described in Fig. 1. At the end of 60 minutes incubation, the reaction was stopped by boiling in SDS and the entire sample was subjected to acrylamide gel electrophoresis in 20% cross-linked gels (13). The gels were sliced in 2 mm slices, extracted and counted (11). Slice 1 is the tracking dye front. The large number of counts at the front is unreacted CDP-[^{3}H]-glycerol. ▲, Reaction mixture contained 2.5 nmoles of CDP-[^{3}H]- glycerol (10^6 dpm) and 200 nmoles of UDP-GlcNAc, O, UDP-GlcNAc was omitted; ●, 5 µg/ml of Tunicamycin were added to the complete reaction mixture.

detectable level of the UDP-GlcNAc-polyribitol phosphate glycosyl transferase, and the polymeric material synthesized from UDP-GlcNAc is not the result of GlcNAc transfer to endogenous polyribitol phosphate.

When CDP-[^{3}H]-glycerol is incubated with the membrane preparation, we observe incorporation of [^{3}H]-glycerol into oligomeric material only in the presence of UDP-GlcNAc and this reaction is inhibited by Tunicamycin (Fig. 1). The radioactive material moves as a single compound (compound I) on SDS acrylamide gel electrophoresis (Fig. 2). When membranes are allowed to synthesize this product and are then incubated with CDP-ribitol, the intermediate is converted to a compound of higher molecular weight (compound II), as determined by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate (Fig. 3). If the membranes are incubated with UDP-GlcNAc, CDP-[^{3}H]-glycerol and CDP-ribitol, only the higher molecular weight material is seen and no intermediate accumulates (Fig. 3), suggesting that the rate of formation of the intermediate is rate limiting in this system. If CDP-[^{3}H]-ribitol is used as a label, the only polymeric compound seen on acrylamide gels has the mobility of compound II.

When reaction mixtures in which membranes are incubated with CDP-[^{3}H]-glycerol and UDP-GlcNAc are stopped with ethanol-1 M NH_4 acetate (3/7.5) and filtered, compound I is retained on the filter. Compound I can be eluted from the filters by washing with 70% ethanol. Compound I eluted from filters behave as a single compound on electrophoresis on polyacrylamide gels (Fig. 4). Addition of compound I to membranes in the presence of CDP-ribitol and Triton X-100 results in the formation of compound II (Fig. 4). Compound I can also be extracted directly from the membranes after incubation with CDP-[^{3}H]-glycerol and UDP-GlcNAc with 70% ethanol. Membranes are incubated with CDP-[^{3}H]-glycerol, UDP-GlcNAc and ATP. The nucleotides are removed at the end of the incubation by repeated centrifugation and washing, and the membrane pellet containing

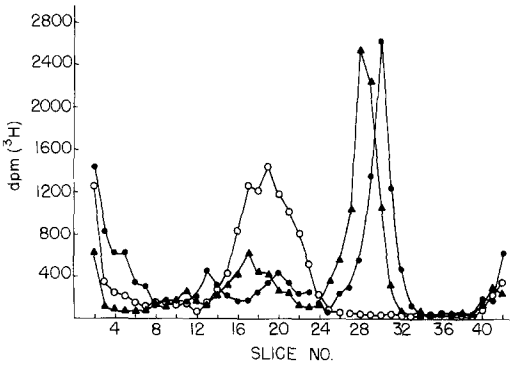

Fig. 3. Addition of polyribitol phosphate to glycerol labelled intermediate. The reaction mixture contained 2.5 nmoles (10^6 dpm) CDP-[^{3}H]-glycerol, 200 nmoles of UDP-GlcNAc. At the end of 30 minutes the incorporation of radioactive glycerol-P was stopped by the addition of 25 nmoles of cold CDP-glycerol and 5 μg/ml of Tunicamycin and incubation was continued for an additional 60 min. -O-, no additions during second incubation. -●-, with 25 nmoles of CDP-ribitol during second incubation. ▲, a reaction mixture to which CDP-ribitol was added at the beginning of the incubation. The reactions were run on SDS acrylamide gels as in Fig. 2 with internal standards of ^{14}C-lipoteichoic acid. The peak of the internal standards was O - slice 22, ● - slice 22, and ▲ - slice 21. In a duplicate experiment (not shown) where CDP-[^{3}H]-ribitol was used as a label, the peak of polyribitolphosphate was at slice 30.

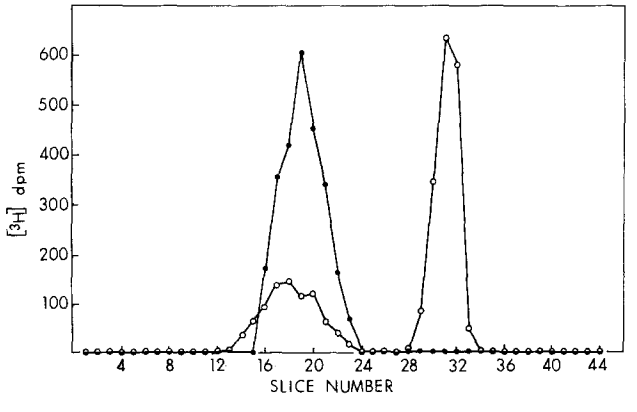

Fig. 4. Conversion of compound I to compound II. Compound I was obtained by extraction of membranes with 70% ethanol as described in the text. It was incubated with S. aureus 52-A2 membranes in 0.2% Triton either in the presence (O) or absence (●) of 25 nmoles of CDP-ribitol for 1 hour. The reactions were then subjected to polyacrylamide gel electrophoresis.

compound I is extracted with 70% ethanol at room temperature.

Compound II is retained on filters both in 30% and 70% ethanol, and

this difference in solubility can be used as an assay for the conversion of compound I to compound II.

We conclude from these and previous observations that the membrane in preparation synthesizes an intermediate which has the general structure

$$X\text{-}(GluNAc)_n - (Glycerol\ P)_m$$

which in the presence of CDP-ribitol is converted to

$$X\text{-}(GlcNAc)_n - (Glycerol\ P)_m - (Ribitol\ P)_{30}.$$

In separate experiments (data not shown) carried out with UDP-[^{3}H]-N-acetyl muramyl pentapeptide, we have seen no evidence for the presence of N-acetyl-muramic acid in either of the two intermediates in teichoic acid biosynthesis.

From the stoichiometry between ribitol and glycerol incoporation in a number of experiments, we find m to be 1.5-2.0 glycerol-P units/chain. We have also analyzed the teichoic acid glycan complex obtained by lysostaphin digestion of whole cell walls and from the excess of glucosamine over muramic acid in this complex, we conclude that n = 1.5-2.0. The structure of X has not yet been determined. The inhibition by Tunicamycin suggests that X may be undecaprenol phosphate.

Previous work in this laboratory has shown that the polyribitol polymerase can catalyze the reaction (10-12)

$$30\ CDP\text{-ribitol} + LTC \rightarrow LTC\text{-}(ribitol\ P)_{30} + 30\ CMP$$

where LTC is lipoteichoic acid carrier (or lipoteichoic acid). We have had great difficulty in separating LTC-(ribitol P)$_{30}$ from X-(GlcNAc)$_n$-(Glycerol P)$_m$-(Ribitol P)$_{30}$ and thus cannot be certain at the moment whether LTC-(ribitol P)$_{30}$ is an intermediate in the synthesis of this latter compound or whether LTC is an analogue of the normal acceptor for the polyribitol phosphate polymerase, especially since in each case the linkage appears to be a phosphodiester linkage between polyribitol phosphate and glycerol-P (4,12).

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