BIOSYNTHESIS OF THE UNIT THAT LINKS TEICHOIC ACID TO THE BACTERIAL WALL: INHIBITION BY TUNICAMYCIN

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1. Introduction

The walls of Gram-positive bacteria contain acidic polysaccharides and teichoic acids that are covalently linked through phosphodiesters to muramic acid residues in the peptidoglycan. It has been shown recently that in *Staphylococcus aureus* H [1] and in a mutant strain that lacks *N*-acetylglucosaminyl substituents on its wall teichoic acid [2] a 'linkage unit' that is a trimer of glycerol phosphate is interposed between the phosphate-terminal end of the teichoic acid chain and a muramic acid residue of the peptidoglycan to which it is attached. Evidence for similar linkage units has been found in *Micrococcus* sp. 2102 and in *Bacillus subtilis* W32 [J. Coley and E. Tarelli, unpublished work].

None of the bacteria examined contains a glycerol teichoic acid in its wall, and it has been demonstrated that the glycerol membrane teichoic acids are synthesized from phosphatidylglycerol [3,4]. Nevertheless, we have found [5] that membrane preparations from all of them catalyse the synthesis from CDP-glycerol of material containing a glycerol phosphate oligomer. Maximal synthesis of this product requires the addition of UDP-*N*-acetylglucosamine and the substrate for the synthesis of the backbone of the wall teichoic acid. The product is amphiphilic and appears to consist of the teichoic acid chain linked through the glycerol phosphate oligomer to a lipid acceptor. Bracha and Glaser [6] found that synthesis of the ribitol teichoic acid covalently linked to wall, in a wall-membrane preparation from *S. aureus* H, also required the addition of CDP-glycerol and UDP-*N*-acetylglucosamine. If therefore appears that teichoic acid attached to linkage unit is synthesized in a membrane-bound form before it is transferred to the cell wall.

When poly(ribitol phosphate) is synthesized from labelled CDP-ribitol alone, in membranes of *S. aureus* H, a single product is formed which is poly(ribitol phosphate) attached to lipoteichoic acid carrier (LTC) [7]. We report here that when CDP-glycerol and UDP-*N*-acetylglucosamine are also present, an additional poly(ribitol phosphate) - containing product is obtained. This new material co-chromatographs with the material synthesized from CDP-[^{3}H]glycerol in the presence of CDP-ribitol and UDP-*N*-acetylglucosamine. The antibiotic tunicamycin inhibits incorporation of glycerol phosphate from CDP-glycerol into membrane preparations and also inhibits formation of the second poly(ribitol phosphate) species. These results can be explained by the earlier suggestion [5] that two different lipid carriers are involved in the synthesis of wall teichoic acid-linkage unit complexes. The inhibitory action tunicamycin suggests that one of these lipid carriers might be a polyisoprenyl phosphate.

2. Methods

CDP-[1-^{3}H]ribitol was synthesized enzymically from [1-^{3}H]ribitol 5-phosphate and CTP using a crude CDP-ribitol pyrophosphorylase preparation from *S. aureus* H [8]. [1-^{3}H]Ribitol 5-phosphate was prepared by reduction of ribose 5-phosphate with potassium [^{3}H]borohydride. The specific activity of the CDP-ribitol was 75 μCi/μmol. Preparation of the other substrates has been described previously [5].

Membranes were prepared from *S. aureus* H dis-

rupted with glass beads as previously described [5]. Membranes of *B. subtilis* W23 were obtained by lysis of the cells with lysozyme as described for *Bacillus licheniformis* [9]. Membranes were suspended in 50 mM Tris–HCl, pH 8.0, containing 5 mM dithiothreitol, at 20 mg dry wt. of membrane/ml.

Unglycosylated poly(ribitol phosphate) was obtained by extraction with dilute alkali [10] from walls of *B. subtilis* W23 mutant M3 that lacked glucosyl substituents on its wall teichoic acid. The walls were the generous gift of Dr A. R. Archibald.

Incorporation of radioactivity into polymeric products was measured on paper as previously described [5]. For ion-exchange chromatography the products were isolated by a different procedure: to the reaction mixture (0.2 ml) was added 0.4 ml of propan-1-ol and the mixture was kept at room temperature for 15 min and then centrifuged at 1000 *g* for 5 min. The resulting pellet was suspended in 0.8 ml of 50% (v/v) propanol for 15 min at room temperature and centrifuged again. Polymeric material was then extracted from the pellet into 0.4 ml of 1% (w/v) Triton X-100 at 60°C for 15 min and insoluble material was removed by centrifugation as before. Samples of the extracts were examined for the absence of residual labelled substrates by paper chromatography. Ion-exchange chromatography was carried out on a column (1.6 X 35 cm) of DEAE-cellulose (Whatman, DE52) equilibrated in 0.1% (w/v) Triton X-100 containing 5 mM disodium EDTA (pH 4.5). The sample was applied in 2.0 ml of 0.5% (w/v) Triton X-100 and material was eluted with a linear gradient (200 ml) of 0 to 1.0 M sodium chloride in the equilibration solution at a flow rate of 60 ml/h; 2.0 ml fractions were collected.

Tunicamycin (Lot. No. T-12-06) was the generous gift of Professor G. Tamura.

For assay of the glycosylation of added poly(ribitol phosphate) the incubation mixtures were as follows: *S. aureus* H membrane (2 mg dry wt. membrane), magnesium acetate (40 mM), UDP-[U-^{3}H]-*N*-acetylglycosamine (0.025 μCi, 0.2 mM) or *B. subtilis* W23 membrane (2.5 mg dry wt membrane), magnesium acetate (40 mM), UDP-[U-^{3}H]glucose (0.67 μCi, 0.38 mM). Poly ribitol phosphate (containing 0.17 μmol phosphorus) was added where indicated. The total volume was 0.13 ml. Incorporation of radioactivity into polymeric products at 37°C during 1 h was measured by paper chromatography as previously described [5].

All measurements of radioactivity were carried out as previously described [5].

3. Results and discussion

Figure 1 shows the separation of the labelled polymeric products from membranes of *S. aureus* H obtained from CDP-[^{3}H]ribitol and UDP-*N*-acetylglucosamine in the presence and absence of CDP-glycerol. Formation of the major product obtained in the absence of CDP-glycerol (R1) was unaffected by the omission of UDP-*N*-acetylglucosamine, but this nucleotide greatly stimulated the formation of the additional component (R2) that was synthesized in the presence of CDP-glycerol. Both R1 and R2 gave radioactive anhydroribitol and ribitol phosphates, with smaller amounts of ribitol, on acid hydrolysis. These are the hydrolysis products expected from poly(ribitol phosphate). Radioactive polymer synthesized from CDP-[^{3}H]glycerol in the presence of CDP-ribitol and UDP-*N*-acetylglucosamine [5] was

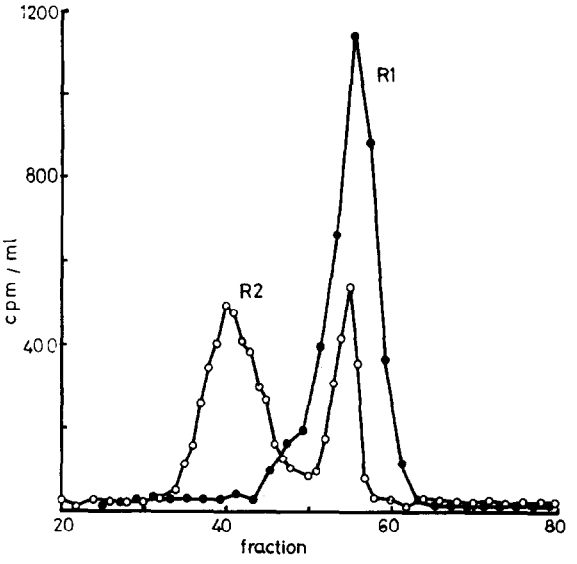

Fig.1. Ion-exchange chromatography of radioactive poly(ribitol phosphate) synthesized in the presence o——o, and absence •——• of CDP-glycerol. 1 ml samples of each fraction were counted for radioactivity. The counts shown have not been corrected for background or variation in counting efficiency.

eluted from the column as a single component coincident with R2; it is probably therefore that R2 is identical with the polymer labelled from CDP-glycerol.

Synthesis of polymer from CDP-[^{3}H]glycerol in the presence of CDP-ribitol and UDP-*N*-acetylglucosamine was strongly inhibited by the antibiotic tunicamycin in both *S. aureus* and *B. subtilis*. In *S. aureus* H 50% inhibition was caused by 1 μg of antiobiotic/ml. At 3 μg of antibiotic/ml glycerol incorporation was inhibited by 80%, and at concentrations above 10 μg antibiotic/ml inhibition was about 90%. 3 μg of tunicamycin/ml did not inhibit incorporation of ribitol phosphate from CDP-ribitol alone, but caused 40% inhibition when CDP-glycerol and UDP-*N*-acetylglucosamine were also present (table 1). Figure 2 shows that almost all the inhibition of ribitol incorporation in the presence of CDP-glycerol and UDP-*N*-acetylglucosamine was because of inhibition of the synthesis of component R2 and not of R1. The residual material in R2 was eluted slightly later than the bulk of the material in R2 from the uninhibited preparation. This may indicate that R2 is heterogeneous, but we have been unable to obtain any further resolution within the peak.

Qualitatively similar results were obtained using a membrane preparation from *B. subtilis* W23 (table 1), which contains a glucosylated poly(ribitol phosphate) in its wall. On storage, frozen at −20°C, the membrane preparation gradually lost the ability to synthesize R2 during a period of 2 months, but retained full activity for synthesizing R1. This ageing effect was responsible for the discrepancy between the proportions of R1 and R2 in the two experiments with CDP-glycerol and UDP-*N*-acetylglucosamine shown in table 1.

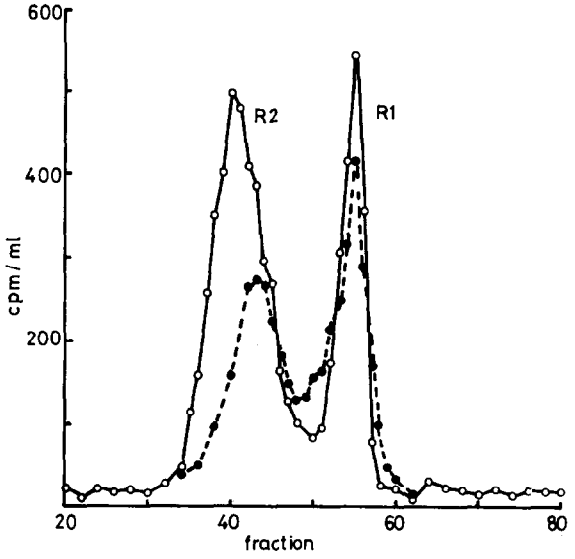

Fig.2. Ion-exchange chromatography of radioactive poly(ribitol phosphate) synthesized in the presence of UDP-*N*-acetylglucosamine and CDP-glycerol. o——o no tunicamycin; •- - -• 3 μg tunicamycin/ml.

One notable difference between the behaviour of the two bacterial systems was in the effect of CDP-glycerol together with UDP-*N*-acetylglucosamine on the total incorporation of label from CDP-ribitol into polymeric products. In *B. subtilis* membranes the two added nucleotides increased overall poly(ribitol phosphate) synthesis by about 77%, whereas they inhibited in *S. aureus* by about 30%.

Fiedler and Glaser [7] obtained a fraction from triton-solubilized membranes of *S. aureus* H that catalysed the synthesis of poly(ribitol phosphate) from CDP-ribitol in the presence of added acceptor. The acceptor had the properties of a membrane teichoic acid and was named lipoteichoic acid carrier (LTC). A single polymeric product was obtained that consisted of a complete poly(ribitol phosphate) chain comprising about forty ribitol phosphate residues linked through its phosphate terminal end to an unknown position on LTC. It is likely that the major component R1 of the poly(ribitol phosphate) synthesized from CDP-ribitol alone in our systems is also poly(ribitol phosphate)-LTC. In the presence of CDP-glycerol and UDP-*N*-acetylglucosamine an additional ribitol-containing polymer (R2) is formed, which appears to be identical with the polymer synthesized from labelled CDP-glycerol in the presence of UDP-*N*-acetylglucosamine and CDP-ribitol. We believe that this product consists of a poly(ribitol phosphate) chain linked through its terminal phosphate to the tri(glycerol phosphate) linkage unit described by Heckels, Archibald and Baddiley [2] which is in turn linked to an unidentified lipid acceptor [5]. Although it seems probable that the poly(ribitol phosphate) moiety of R2 originated in R1, we have no direct evidence to confirm this.

Tunicamycin completely inhibited incorporation from CDP-[^{3}H]glycerol into R2, but only partly inhibited incorporation from CDP-[^{3}H]ribitol, in *S. aureus* H. It therefore appears that tunicamycin

Table 1
Incorporation into polymers from CDP-[^{3}H]ribitol and CDP-[^{3}H]glycerol by membrane preparations from *B. subtilis* W23 and *S. aureus* H

Bacterium	Substrates	Total ^{3}H in polymers (cpm)	% Total [^{3}H]Polymer R1	R2
B. subtilis W23 Fresh membranes	CDP-[^{3}H]ribitol	4578	>90	< 10
	CDP-[^{3}H]ribitol CDP-glycerol UDP-*N*-acetylglucosamine	7124	64	36
Aged membranes	CDP-[^{3}H]ribitol CDP-glycerol UDP-*N*-acetylglucosamine	5760	23	77
	as above + tunicamycin	3908	43	57
S. aureus H	CDP-[^{3}H]ribitol	7208	>90	< 10
	CDP-[^{3}H]ribitol CDP-glycerol UDP-*N*-acetylglucosamine	5016	35	65
	as above + tunicamycin	3010	53	47
	CDP-[^{3}H]glycerol	235	not detectable	
	CDP-[^{3}H]glycerol UDP-*N*-acetylglucosamine CDP-ribitol	5540	–	100
	as above + tunicamycin	466	not detectable	

inhibits the synthesis of the tri(glycerol phosphate)-acceptor unit that is dependent upon UDP-*N*-acetylglucosamine, but does not inhibit synthesis of R1. Possibly the membrane contained sufficient endogenous linkage unit-acceptor to allow some synthesis of R2 in the absence of CDP-glycerol and UDP-*N*-acetylglucosamine; synthesise of R2 from this material would not be inhibited by tunicamycin.

Tunicamycin is known to inhibit the synthesis of a polyisoprenyl pyrophosphate *N*-acetylglucosamine that is involved in the biosynthesis of a yeast glycoprotein [11] and a mammalian cell surface glycoprotein [12]. Bettinger and Young [13] and Tamura et al. [14] have also demonstrated that it inhibits the biosynthesis of peptidoglycan in bacteria, but have obtained contradictory evidence about the precise site of action of the antibiotic. In membranes of *Bacillus subtilis* 168 tunicamycin at a concentration of 15 μg/ml inhibited synthesis of the first lipid intermediate, polyisoprenyl pyrophosphate *N*-acetylmuramyl pentapeptide by less than 10% [13] and Bettinger and Young therefore concluded that the antibiotic inhibited the addition of *N*-acetylglucosamine to this lipid to form the disaccharide lipid intermediate *N*-acetylglucosaminyl-*N*-acetylmuramyl pentapeptide pyrophosphate polyisoprenol. On the other hand in a cell-free membrane preparation from *Micrococcus lysodeikticus* tunicamycin at 50 μg/ml inhibited synthesis of the first lipid intermediate by 83% but was far less inhibitory towards the synthesis of the disaccharide lipid [14]. Although the precise specificity of the antibiotic is therefore uncertain, the evidence indicates that at the low concentrations used in our experiments it is an inhibitor of reactions between *N*-acetylhexosamine-containing nucleotides and lipids containing a polyisoprenyl phosphate moiety [12]. Tunicamycin did not inhibit the glycosylation of exogenous poly(ribitol phosphate) either by

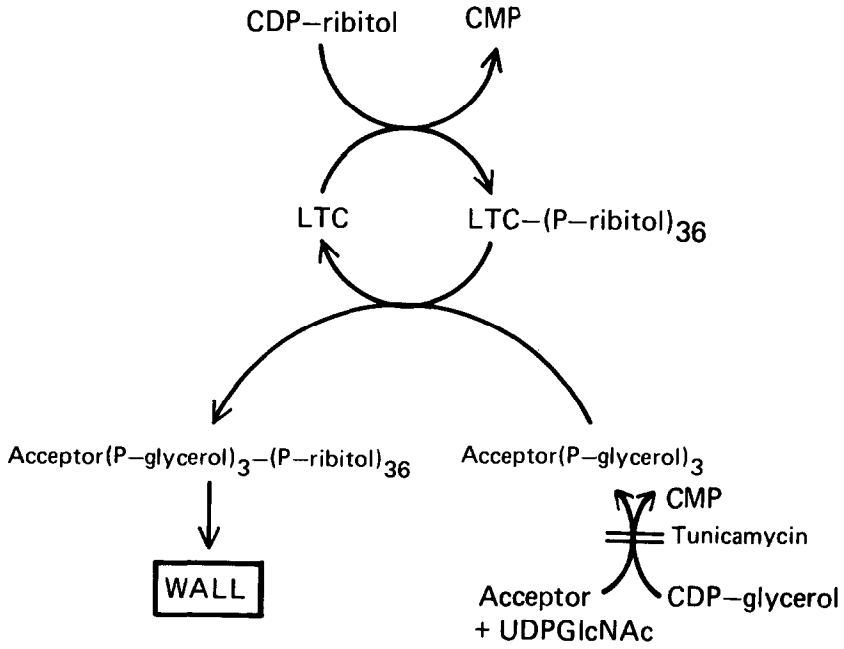

Fig.3. Suggested scheme for the assembly of teichoic acid-linkage unit.

UDP-*N*-acetylglucosamine in membranes of *S. aureus* H or by UDP-glucose in membranes of *B. subtilis* W23. Therefore, the appearance of tunicamycin-sensitive material in peak R2 is not due to the synthesis of the glycosylated derivative of R1 in either case.

Conclusions from these studies are summarized in the scheme (fig.3) for the biosynthesis of wall teichoic acid-linkage unit and its transfer to peptidoglycan to form wall material. Two different acceptors are required; one of these (LTC) is identical to or similar to the membrane teichoic acid and this accepts ribitol phosphate residues from CDP-ribitol to build up the main chain of the wall teichoic acid. The synthesis of the poly(ribitol phosphate)-LTC (peak R1) is not inhibited by tunicamycin. The second acceptor occurs in the butanol soluble fraction from membranes [5] and receives three glycerol phosphate residues from CDP-glycerol to form the linkage unit. The teichoic acid main chain is transferred from poly(ribitol phosphate)-LTC to this lipid containing the linkage unit to give a water-soluble amphiphilic product (R2), the formation of which is strongly inhibited by tunicamycin. Thus R2 comprises poly(ribitol phosphate)-linkage unit–lipid acceptor, and it is this that participates in the attachment of teichoic acid to peptidoglycan. As tunicamycin powerfully inhibits the synthesis of R2, which is dependent upon UDP-*N*-acetylglucosamine, it is likely that the synthesis requires a polyisoprenyl phosphate derivative. This is in agreement with the previous suggestion [5] that the second lipid acceptor might be the polyprenyl pyrophosphate *N*-acetylmuramyl pentapeptide *N*-acetylglucosamine intermediate in peptidoglycan synthesis, although other possible polyisoprenyl phosphate containing *N*-acetylglucosamine have not been excluded.

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