BIOSYNTHESIS OF THE LINKAGE UNIT JOINING PEPTIDOGLYCAN TO POLY(*N*-ACETYLGLUCOSAMINE 1-PHOSPHATE) IN WALLS OF *MICROCOCCUS VARIANS* ATCC 29750

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1. Introduction

The cell wall of *Micrococcus varians* ATCC 29750 contains poly(*N*-acetylglucosamine 1-phosphate) [1]. This polymer, like the teichoic acids of several other Gram-positive bacteria, is joined to muramic acid in the peptidoglycan via an oligomer consisting of *N*-acetylglucosamine phosphate and 3 glycerol phosphate residues [2–5]. This linkage unit is synthesized from UDP-*N*-acetylglucosamine (UDP-GlcNAc) and CDP-glycerol through a series of intermediates containing polyprenyl phosphate [6–8]. The first step in the synthesis of the linkage unit is the formation of polyprenyl pyrophosphate *N*-acetylglucosamine, and this is inhibited by the antibiotic tunicamycin [9–13]. Sequential addition of glycerol phosphate residues from CDP-glycerol leads to the synthesis by membrane preparations from *Staphylococcus aureus* H and *M. varians* of two further lipids, containing respectively 1 and 2 glycerol phosphates, which have been isolated and characterized [6]. A scheme for the biosynthesis of linkage unit attached to poly(ribitol phosphate) in *S. aureus*, or poly(*N*-acetylglucosamine-1-phosphate) in *M. varians*, involving these 3 lipids (identified as lipids I, II and III, respectively) has been presented [6].

The origin of the third glycerol phosphate residue in the linkage was not clear. The synthesis by membranes of either *S. aureus* or *M. varians* of a polyprenyl phosphate intermediate containing 3 glycerol phosphate residues has not yet been demonstrated. However, if the addition of glycerol phosphate to lipid III is rate-limiting for synthesis and attachment of linkage unit, then the intermediate might occur in such small amounts that it would not be detected.

Alternatively, the third glycerol phosphate residue might not be derived from CDP-glycerol, for example, if it was transferred from lipoteichoic acid carrier (LTC) [6]. The structure of LTC is unclear, but it probably contains glycerol phosphate [14]. In this paper we present evidence that in *M. varians* all 3 glycerol phosphate residues in the linkage unit are derived from CDP-glycerol.

Nomenclature: Micrococcus varians ATCC 29750 was previously described as *Micrococcus* sp. 2102

2. Methods

CDP-glycerol, CDP-[2-^{3}H]glycerol and [β-^{32}P]CDP-glycerol were prepared as in [6,15,16]. [β-^{32}P]CDP-[2-^{3}H]glycerol was prepared by mixing appropriate amounts of the singly-labelled substrates to give an isotopic ratio (^{3}H : ^{32}P) of about 4:1. This doubly-labelled material was repurified by paper chromatography [16] and assayed immediately before use. Cells of *Micrococcus varians* ATCC 29750 were grown and membranes prepared therefrom as in [6,17]. Incubation conditions, paper chromatography, electrophoresis, acid and alkaline hydrolysis, treatment with alkaline phosphatase and measurement of radioactivity were as in [6]. Chromatography of products was carried out on a column (1.6 × 50 cm) of Sephadex G-50 eluted with pyridinium acetate (25 mM, pH 6.5) at 40 ml/h flow rate, collecting 4 ml fractions.

An authentic sample of bis(glycerophosphoryl)-glycerol was obtained by deacylation of cardiolipin (Sigma Chemical Co., St Louis) with sodium methoxide [18]. Glycerol diphosphate was the generous gift of Dr J. Coley.

Following separation of incubation mixtures by paper chromatography, polymeric material remaining at the origin was extracted from the paper by hydrolysis with NaOH (0.5 M, 2 h, 20°C) [6]. The extracted polymer was subjected to chromatography on Sephadex G-50 and the excluded material (fractions 9–11) hydrolysed with 0.1 M HCl (100°C, 10 min) to degrade the poly(*N*-acetylglucosamine 1-phosphate) and release the tri(glycerol phosphate) moiety of the linkage unit as in fig.1. Treatment with alkaline phosphatase and purification by chromatography on Sephadex G-50, followed by paper chromatography and electrophoresis concomitantly with authentic materials, yielded bis(glycophosphoryl)glycerol.

Using a modification [19] of the Barry degradation [20] bis(glycerophosphoryl)glycerol was degraded to glycerol diphosphate by oxidation with periodate and treatment with *N,N*-dimethylhydrazine. The glycerol diphosphate was purified by paper chromatography and electrophoresis.

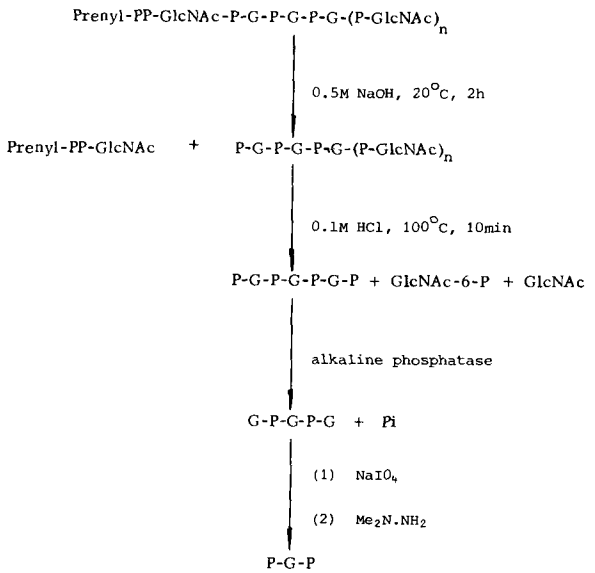

Fig.1. Scheme for the degradation of the poly(*N*-acetylglucosamine 1-phosphate)-linkage unit complex. G represents glycerol; GlcNAc, *N*-acetylglucosamine.

3. Results

Incorporation of CDP-[2-^{3}H]glycerol into lipids and polymer at various incubation temperatures showed that lower temperatures favoured the accumulation of the lipid intermediates, whereas at higher temperatures a relatively greater amount of labelled polymeric material was formed (fig.2).

The hypothesis that the lipids in [6] are successive intermediates in the synthesis of the linkage unit attached to poly(*N*-acetylglucosamine 1-phosphate) in *M. varians* was confirmed in an experiment in which 1 ml membrane suspension was incubated at 30°C for 10 min with UDP-GlcNAc and CDP-[2-^{3}H]-glycerol, then washed free of substrates by the addition of 40 ml ice-cold Tris–HCl (0.05 M, pH 8.0) and

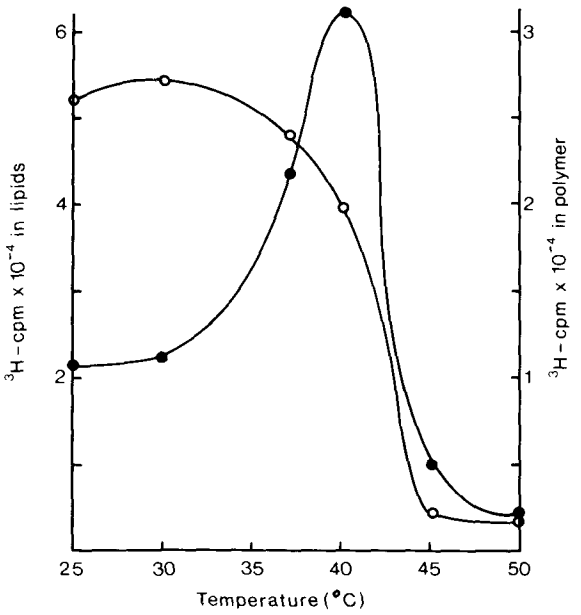

Fig.2. Effect of incubation temperature on the incorporation of CDP-[2-^{3}H]glycerol into lipids (○) and polymer (●). Following incubation at the appropriate temperature for 20 min, reactions were terminated by the addition of 1 ml 20% (v/v) ethanol, the membranes collected by centrifuging (8000 × *g* for 5 min), then washed again with 1 ml 20% ethanol to remove residual radioactive substrate. Lipids were extracted from the particulate fraction using 70% ethanol (2 × 0.5 ml) as in [10], and the residual pellet containing the polymeric material was resuspended in 1 ml 0.1% dodecyl sulphate. Radioactivity in lipid and polymer fractions was determined as in [6].

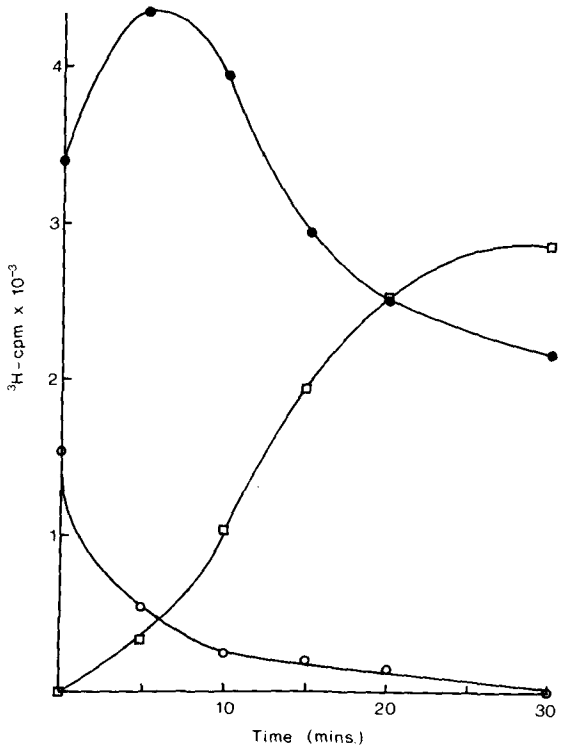

Fig.3. The fate of radioactive lipids formed in membranes which had been pre-incubated with CDP-[2-^{3}H]glycerol and UDP-GlcNAc. The washed membranes were reincubated with unlabelled substrates and samples taken at intervals. Radioactivity in the products was measured following their separation on paper chromatograms as in [6]. Lipid II (○), lipid III (●), increase in radioactivity in polmer from $t = 0$ (□).

centrifuging at 50 000 X *g* for 30 min to recover the membrane. Paper chromatography revealed only a trace of residual labelled nucleotide in the washed membranes, whereas radioactivity had been incorporated into lipids II, III and polymer. The membrane pellet was resuspended to 1 ml in the same buffer and incubated again with UDP-GlcNAc and CDP-glycerol. This incubation was done at 37°C to favour synthesis of poly(*N*-acetylglucosamine 1-phosphate) attached to linkage unit. Samples (0.15 ml) were taken at intervals and the radioactive polymer and lipids separated by paper chromatography. The results (fig.3) show that labelled lipid II, initially formed in the pre-incubated membranes, decreased on re-incubation with unlabelled substrates. Concomitantly, radioactivity in lipid III increased, and subsequently fell as radioactivity was transferred to polymer.

To study the further metabolism of the lipid III fraction to polymer, 1 ml membrane suspension was incubated with UDP-GlcNAc and CDP-[2-^{3}H]glycerol for 30 min at 30°C. Following chromatography of the incubation mixture, material from the region of the chromatogram containing lipid III was eluted with 70% (v/v) ethanol. The extract was evaporated to dryness under N_2 at 25°C and redissolved in water (100 μl) containing Triton X-100 (1.5% w/v). Portions (20 μl, 10^5 dpm) of this solution containing lipid III were then incubated with membranes at 37°C in the presence of UDP-GlcNAc, CDP-glycerol, or both of these substrates. Following chromatography the amount of radioactivity that had been incorporated into polymer was determined (table 1). Incorporation of radioactivity from lipid III into polymer was clearly stimulated in the presence of CDP-glycerol. Greatest stimulation was found when CDP-glycerol and UDP-GlcNAc were both included.

The pattern of incorporation of CDP-glycerol into the tri(glycerol phosphate) moiety of the linkage unit to which polymer had been attached was examined using [β-^{32}P]CDP-[2-^{3}H]glycerol. Membrane suspension, 1 ml, was incubated with the doubly-labelled substrate and UDP-GlcNAc. The isotope ratio (^{3}H : ^{32}P) in substrate, alkali-extracted polymer and bis(glycerophosphoryl)glycerol derived therefrom (see section 2) was

Table 1
Incorporation of radioactivity from exogenous [^{3}H]lipid III into polymer by *M. varians* membranes

Unlabelled substrates added	Polymer (cpm)
None	640
UDP-GlcNAc	720
CDP-glycerol	1760
CDP-glycerol and UDP-GlcNAc	3120
None boiled membrane suspension	170

Radioactive lipid III was isolated from membranes that had been incubated with CDP-[2-^{3}H]glycerol and UDP-GlcNAc at 30°C. Portions (10^5 dpm) of this lipid were then reincubated with membranes and unlabelled substrates at 37°C as shown. Radioactivity incorporated into polymer was measured following separation of the incubation mixtures by paper chromatography

Table 2
Incorporation of β-[^{32}P]CDP-[2-^{3}H]glycerol into polymeric material by *M. varians* membranes

	^{3}H (dpm)	^{32}P (dpm)[a]	Isotope ratio (^{3}H : ^{32}P)	Isotope ratio relative to substrate
(1) β-[^{32}P]CDP-[2-^{3}H]glycerol	10 262	2665	3.85	1:1
Alkali-extracted polymer	20 730	5830	3.56	0.92:1
Bis(glycerophosphoryl)glycerol	15 765	2820	5.59	2.90:2
Glycerol diphosphate	13 005	7322	1.78	0.92:2
(2) β-[^{32}P]CDP-[2-^{3}H]glycerol	14 337	2233	6.42	1:1
Alkali-extracted polymer	21 146	3194	6.62	1.03:1
Bis(glycerophosphoryl)glycerol	39 362	4170	9.44	2.94:2
Glycerol diphosphate	11 340	3750	3.02	0.94:2

[a] Corrected for decay

The tri(glycerol phosphate) moiety of the linkage unit was isolated as in section 2 (fig.1), and the isotope ratios of the various products determined. Results for 2 separate experiments are given

determined. Finally, bis(glycerophosphoryl)glycerol was degraded to glycerol diphosphate, and the isotope ratio of the purified glycerol diphosphate also determined. The results of 2 such experiments are presented in table 2.

4. Discussion

The results shown in fig.3, of the pulse chase experiment confirm that lipids II and III are sequential intermediates on the pathway of biosynthesis of linkage unit and its subsequent incorporation into polymeric material. A fourth lipid containing 3 glycerol phosphate residues was not found. However, CDP-glycerol greatly stimulated the incorporation of radioactivity from exogenous lipid III into polymer by the membranes (table 1). This suggests that CDP-glycerol is the precursor of the third glycerol phosphate residue in the linkage unit. The greatest stimulation was observed when both CDP-glycerol and UDP-GlcNAc (the precursor of the main polymer chain in *M. varians*) were included. The membrane preparation undoubtedly contains a little endogenous poly-(*N*-acetylglucosamine 1-phosphate) which allows a corresponding synthesis of the linkage unit-polymer to proceed in the absence of UDP-GlcNAc.

The synthesis of linkage unit attached to polymer from [β-^{32}P]CDP-[2-^{3}H]glycerol, and its subsequent degradation to bis(glycerophosphoryl)glycerol and glycerol diphosphate (table 2) confirms that all 3 glycerol phosphate residues are derived from CDP-glycerol. The isotope ratios (^{3}H : ^{32}P) found in the isolated bis(glycerophosphoryl)glycerol (3:2) and glycerol diphosphate (1:2) can only arise if all 3 glycerol phosphate residues in the linkage unit are uniformly labelled. If the third glycerol phosphate were derived from a source other than CDP-glycerol the linkage unit would contain isotope in only two of its three glycerol phosphates and the values for the isotope ratios would be different; the bis(glycerophosphoryl)glycerol would contain one unlabelled glycerol and one unlabelled phosphate and have an isotope ratio of 2:1. Moreover, the glycerol diphosphate would contain an unlabelled phosphate and have the same isotope ratio as the substrate. These conclusions are summarised in fig.4.

When bis(glycerophosphoryl)glycerol was isolated by degradation of the linkage unit-polymer, a little glycerophosphorylglycerol and free glycerol were also formed. The amounts varied (20–35% of the total glycerol) but it is unlikely that they arose solely by hydrolysis of the tri(glycerolphosphate) during the acid and alkali treatment. It is possible that the membrane preparation can accomplish the transfer of completed polymer chains to incomplete linkage unit lipid intermediates (lipids II and III). Such a mechanism would also explain the formation of small

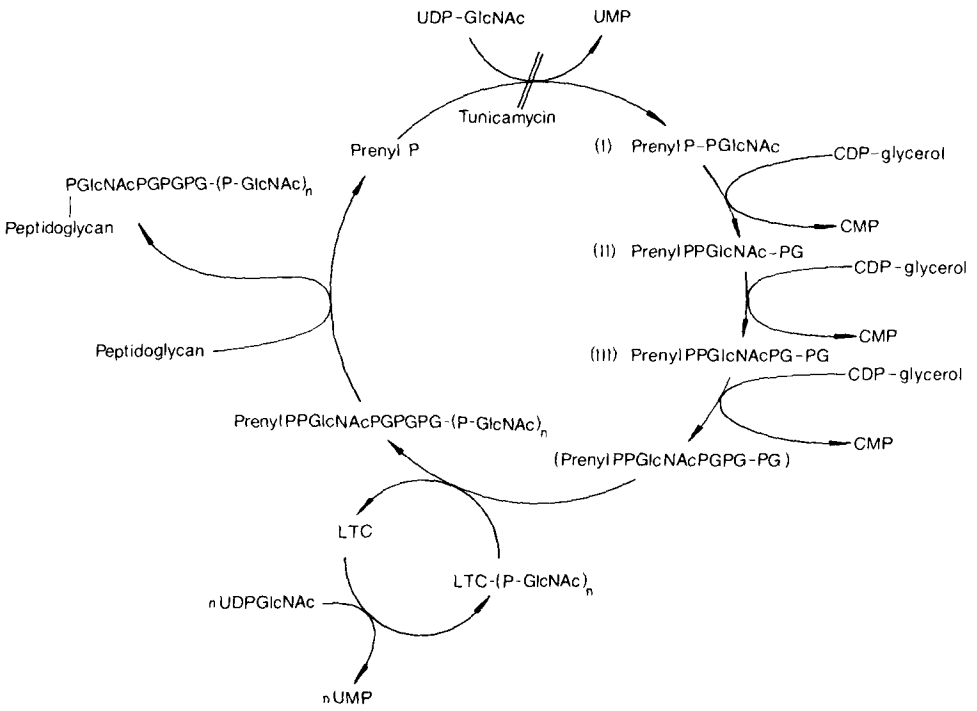

Fig.4. Scheme for the assembly of poly(*N*-acetylglucosamine 1-phosphate)-linkage unit in *M. varians*. PG represents a glycerophosphoryl residue; GlcNAc, *N*-acetylglucosamine. The lipids (I, II and III) have been characterized [6]. Polyprenyl phosphate bearing the completed linkage unit but poly(*N*-acetylglucosamine 1-phosphate) has not been isolated.

amounts of polymer from exogenous lipid III in the absence of added CDP-glycerol (table 1). A similar effect has been observed in studies on the in vitro glycosylation of proteins by polyprenyl intermediates in *Saccharomyces cerevisiae* [21].

Acknowledgement

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