A LINKAGE UNIT JOINING PEPTIDOGLYCAN TO TEICHOIC ACID IN *STAPHYLOCOCCUS AUREUS* H

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1. Introduction

Walls of many Gram-positive bacteria are composed principally of teichoic acid and peptidoglycan, and a great deal is now known about the structure and biosynthesis of these polymers. A major unsolved feature has been the way in which these two polymers are attached to each other; consequently there is little information on how they become attached during synthesis and assembly of the wall. It has long been known that teichoic acids are covalently linked to the glycan chain of peptidoglycan and that this linkage probably involves a phosphorylated muramic acid residue of the glycan. However difficulties have been encountered in studies on the precise nature of the way in which these major wall components are associated. In order to simplify such studies we have recently examined a mutant of *Staphylococcus aureus* H [1] that lacks the *N*-acetylglucosaminyl substituents normally present on the ribitol residues of the teichoic acid [2–4]. In addition to poly(ribitol phosphate) walls of this mutant were found to contain an oligomer of glycerol phosphate [1]. This could have been present as a separate component, independently linked to the peptidoglycan, or it could have been interposed between the ribitol teichoic acid and the glycan so as to constitute a 'linkage unit'. The glycerol phosphate oligomer isolated by alkali extraction of walls that had first been oxidized with sodium metaperiodate, so as to destroy and remove the ribitol acid, and then reduced with potassium [^{3}H]borohydride consisted of a chain of three glycerol phosphate residues attached to which was a radioactively labelled ethylene glycol phosphate residue. It seemed possible that this residue had arisen from the phosphate-terminal ribitol of the teichoic acid, which in the intact wall would therefore have been linked to the glycerol phosphate oligomer. However the alternative possibility, that the ethylene glycol phosphate residue had been produced from a glycerol phosphate residue, and that consequently the glycerol phosphate oligomer was present as a separate component in the wall, could not be excluded. The resolution of this crucial point can however be achieved by similar examination of walls of the parent organism, since in this bacterium the ribitol teichoic acid contains *N*-acetylglucosaminyl substituents which modify the periodate oxidation of the ribitol residues in such a way that the above degradation would leave a substituted glycerol rather than ethylene glycol attached to the glycerol phosphate oligomer if the latter were interposed between the ribitol teichoic and the glycan. If, on the other hand, the glycerol phosphate oligomer was not attached to the ribitol teichoic acid, then the products obtained from its degradation would be unaffected by alterations in the structure of the ribitol teichoic acid. We have therefore repeated the degradative procedures using walls of the parent strain, *Staphylococcus aureus* H. The glycerol phosphate oligomer isolated from walls of this organism did not contain radioactive ethylene glycol but did contain a substituted radioactive glycerol phosphate residue. The characterization of this oligomer shows that it is indeed derived from a linkage unit that is interposed between the ribitol teichoic acid and a muramic acid residue in the peptidoglycan.

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2. Methods

Cells of *S.aureus* H were grown and walls prepared as described before for the mutant [1]. The walls were freed from membrane and other contaminants by treatment with trypsin, 80% (w/v) aq. phenol and sodium decyl sulphate. Periodate oxidation, reduction with potassium [^{3}H]borohydride, hydrolysis with *Flavobacter* L 11 enzyme, acid and alkali hydrolysis, and all analytical and chromatographic methods were as described previously [1].

3. Results and discussion

The walls contained 3.3% of phosphorus, most of which was present as ribitol teichoic acid in which *N*-acetylglucosaminyl substituents were attached to each ribitol unit. The average chain length of the teichoic acid was approximately 36 alditol phosphate units, and after oxidation with periodate the walls contained about 10% of the original phosphate, corresponding to four residual phosphates for each teichoic acid chain.

After reduction of the oxidized walls with potassium [^{3}H]borohydride the residual wall material was dissolved by incubation with *Flavobacter* L-11 peptidase [5]. All of the phosphate that had remained in the walls after treatment with periodate was associated with part of the glycopeptide and this material was isolated chromatographically as before. On paper electrophoresis at pH 5.3 this phosphate-containing glycopeptide migrated as a single component with $R_{\text{glycerol 1-phosphate}}$ 0.63; it contained reduced aldehydic groups, hexosamine and phosphate (all of which was present as diester) in the molecular proportions 0.88 : 5.15 : 1.0. The products of its acid hydrolysis included muramic acid phosphate, [^{3}H]-glycerol and its monophosphate, but radioactive ethylene glycol was not produced. Treatment with dilute alkali hydrolysed the phosphate-containing glycopeptide to glycan and a phosphate that was isolated by ion exchange chromatography and preparative paper electrophoresis as before [1]. On paper electrophoresis at pH 5.3 it migrated as a single component, $R_{\text{glycerol 1-phosphate}}$ 1.35, which gave a positive reaction with the Rydon-Smith spray reagent [6], indicative of a substituted amide. After incubation with phosphomonoesterase the phosphate migrated on electrophoresis as a single component with $R_{\text{glycerol 1-phosphate}}$ 1.2 and which, unlike the material examined before dephosphorylation, rapidly gave a purple colour with the periodate–Schiff reagent [7] (a characteristic of formaldehyde production). All of the phosphate present in periodate oxidized walls was therefore present as a single component. On acid hydrolysis the isolated phosphate gave radioactive glycerol, glycerol monophosphates and 2-acetamido-2-deoxyglyceraldehyde together with unlabelled glycerol diphosphate. The structure of the aldehyde was established by reduction of the acid hydrolysate with sodium borohydride, followed by paper chromatography in ethyl acetate–pyridine–acetic acid–water (5 : 5 : 1 : 3, by vol), where the product co-chromatographed with an authentic sample of 2-acetamido-2-deoxyglycerol. The presence of 2-acetamido-2-deoxy-glyceraldehyde in the acid hydrolysate of the phosphate is due to oxidation and reduction of the *N*-acetylglucosaminyl residue attached to the terminal ribitol of the teichoic acid.

The phosphate contained reduced aldehydic groups and phosphate in the molar ratio 0.74 : 1. On incubation with phosphomonoesterase 21% of its phosphate was converted to inorganic phosphate; the product was oxidized with periodate and formaldehyde was formed in the molar ratio of formaldehyde to phosphate of 0.3 : 1. These values are consistent with the structure shown in fig.1; the labelled products would have arisen from hydrolysis of the terminal glycerol residue bearing substituents derived from the *N*-acetylglucosaminyl on the phosphate-terminal ribitol of the teichoic acid. This glycerol residue is attached to a chain containing three glycerol phosphate residues and which terminates in a phosphomonoester residue. The phosphomonoester group is produced by the action of alkali on the phosphate-containing glycopeptide;

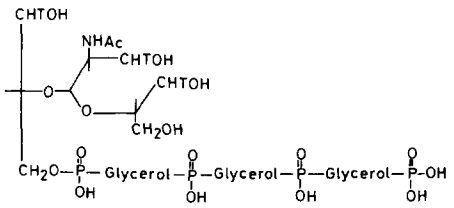

Fig.1. Structure of the phosphate isolated after controlled alkali hydrolysis of the phosphate-containing glycopeptide.

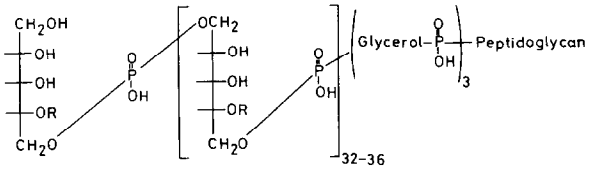

Fig.2. Scheme for the linkage of ribitol teichoic acid to peptidoglycan. Mutant, R = H Parent strains, R = *N*-acetylglucosaminyl.

and since muramic acid phosphate is produced on acid hydrolysis of this glycopeptide but not on acid hydrolysis of the products of alkali cleavage, it follows that the phosphomonoester group is formed by hydrolysis of a phosphodiester linkage between the terminal glycerol residue and muramic acid. The position of substitution of the phosphodiester linkages on adjacent glycerol residues has not been determined; however, the terminal glycerol residue must be attached to the rest of the chain by a phosphodiester linkage to a primary hydroxyl group since formaldehyde is formed on periodate oxidation of the oligomers after enzymic removal of the phosphomonoester group.

No ethylene glycol phosphate was found in the oxidized and reduced walls and, since the radioactive glycerol monophosphate and 2-acetamido-2-deoxyglyceraldehyde originating from the ribitol teichoic acid were associated with the glycerol phosphate oligomer, it follows that the three glycerol phosphate residues are interposed between the ribitol teichoic acid and the peptidoglycan (fig.2).

The establishment of the existence of such a linkage in *S.aureus* strengthens our view that these linkage units might be widespread amongst bacteria. Thus the chemical composition and properties of the walls of a number of unrelated bacteria examined in this laboratory are readily explained on the basis of similar linkage units, e.g. in *Bacillus subtilis* W23 where a ribitol teichoic acid containing glucosyl substituents occurs, in *S.lactis* I3 where the teichoic acid is a polymer of glycerophosphoryl-*N*-acetylglucosamine 1-phosphate and in *Micrococcus* sp. 2102 where the wall polymer comprises units of *N*-acetylglucosamine 1-phosphate. Recent biosynthetic studies (I. C. Hanock and J. Baddiley, submitted for publication) support this widespread occurrence.

Acknowledgement

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