ON THE LINKAGE BETWEEN TEICHOIC ACID AND THE GLYCOPEPTIDE IN THE CELL WALL OF STAPHYLOCOCCUS AUREUS

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Experiments carried out a few years ago on the teichoic acid and glycopeptide in the cell wall of Staphylococcus aureus suggested that their separation in trichloroacetic acid (TCA) might not be due to a simple dissociation of ionic and/or hydrogen bonds. For example, at 3° in 10 % TCA the separation was extremely slow and three weeks were required to solubilize more than 95 % of the teichoic acid. The extraction was markedly temperature dependent and was accelerated with a Q_{10} of between 2 and 3. At 60°, about 95 % of the teichoic acid was solubilized in 10 - 12 hours. These experiments were carried out with strain Copenhagen, but since then, they have been repeated with three other strains (H, Duncan and 3528) with identical results. These data suggested that the dissociation of the two polymers might involve a hydrolytic cleavage of covalent bonds in TCA (Mandelstam and Strominger, 1961). It seemed unlikely, moreover, that ionic bonds could play a major role in the linkage of this teichoic acid with a strong net negative charge (Sanderson et al., 1962) to the glycopeptide, also negatively charged and containing virtually no positively charged groups (Mandelstam and Strominger, 1961; Ghuysen and Strominger, 1963).

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One possibility for the linkage which was considered at that time was a peptide bridge between the glycopeptide and the D-alanine esters in the teichoic acid (Mandelstam and Strominger, 1961). Supporting this possibility was the fact that only a small number of the free amino groups which should exist in the D-alanine ester of the teichoic acid in the cell wall would react with dinitrofluorobenzene. It was recognized, however, that reaction of dinitrofluorobenzene with the insoluble cell wall was a heterogeneous one and could, consequently, have been incomplete (Mandelstam and Strominger, 1961). Moreover, it was then observed by Archibald et al., (1961) that the D-alanine could be released from the teichoic acid in the cell wall without solubilizing the polymer. No evidence, however, has been produced to support the view (Archibald et al., 1961; Baddiley et al., 1962; Critchley et al., 1962) that the teichoic acid is held to the wall of S. aureus only by relatively weak ionic bonds.[1]

The two polymers in the wall of S. aureus can also be separated after solubilization of the walls with hydrolytic enzymes near neutral pH (Ghuysen and Strominger, 1963). Two enzymes were employed for this purpose, an acetylmuramidase which hydrolyzes all of the glycosidic linkages of N-acetylmuramic acid and of N,O-diacetylmuramic acid (Ghuysen and Strominger, 1963) in the cell wall of S. aureus and an amidase which hydrolyzes the amidic linkages between acetylmuramic acid (or diacetylmuramic acid) and L-alanine. After solubilization of the wall by the first enzyme, the glycopeptide with a slight negative

[1] It was originally shown with S. aureus, strain H (Park and Hancock, 1960) and later with Lactobacillus arabinosus and Bacillus subtilis (Archibald et al., 1961) that the glycopeptide remaining after removal of teichoic acid with TCA has the appearance of cell wall in the electron microscope. We have confirmed this fact with S. aureus, strain Copenhagen. The teichoic acid cannot, therefore, contribute to the structural framework of the wall, but these observations do not preclude attachment of the teichoic acid by covalent linkages at the surface or elsewhere in the structure.

charge hardly moved from the origin and was readily separated by electrophoresis at pH 3.8 from a teichoic acid-glycopeptide complex which has a strong negative charge and moved rapidly toward the anode. The glycopeptide moiety of the complex could not be separated from the teichoic acid by electrophoresis at pH 1.5, 2.1 or 5.8 or by repeating the electrophoresis at pH 3.8. Moreover, the complex had a lower electrophoretic mobility than the teichoic acid prepared through the action of TCA on the cell walls.[2] Similarly, the teichoic acid-glycopeptide complex behaved as a single material on chromatography on Ecteola-cellulose (although a mixture of glycopeptide and acid-extracted teichoic acid were readily separated by this technique)[3] and on sedimentation in the ultracentrifuge.

The teichoic acid-glycopeptide complex contained the following constituents, expressed as moles per mole of phosphate: Acetylglucosamine 1.1, acetylmuramic acid 0.07, glutamic acid 0.097, lysine 0.076, glycine 0.54, total alanine 0.62, D-alanine 0.51 and L-alanine 0.089. Most of the D-alanine was present as a labile ester (0.49 mole per mole of phosphate). After removal of ester alanine at pH 8.5, the complex of alanine-free teichoic acid and glycopeptide remained non-dissociable by electrophoresis and by chromatography on Ecteola-cellulose. These and other data (Ghuysen and Strominger, 1963) indicated that there was one disaccharide of acetylglucosamine and acetylmuramic acid and one peptide subunit (composed of 5 glycine, 2 alanine, 1 glutamic acid and 1 lysine residue) per 8 - 10 repeating

[2] For example, in 0.03 N trichloroacetic acid at pH 1.5, the mobilities, in cm per hour at a voltage gradient of 30 v/cm, were: acid extracted teichoic acid, 5.8; teichoic acid-glycopeptide complex, 3.4; and native teichoic acid (see below), 8.5.

[3] This experiment was carried out by Dr. Donald Tipper.

units of the teichoic acid (containing 1 ribitol phosphate, 1 acetylglucosamine and 0.5 D-alanine (Sanderson et al., 1962)). Although a formaldehyde end group was obtained on periodate oxidation, surprisingly this complex contained no phosphomonoester end groups (less than 1 phosphomonoester group per 1000 organic phosphate residues, compared to a value of about 1 phosphomonoester per 14 phosphates in the teichoic acid prepared through the action of TCA (Sanderson et al., 1962)).[4] The glycopeptide moiety was about 10 % of the weight of the complex and represented 20 - 30 % of the glycopeptide in the wall. A similar material, termed polysaccharide A, has been obtained in low yield from whole cells of another strain of S. aureus through the action of autolytic enzymes (Haukenes, 1962). This material lacked the D-alanine ester groups which were presumably lost during the period of autolysis.

Further treatment of the teichoic acid-glycopeptide complex with the amidase (Ghuysen and Strominger, 1963) resulted in liberation of a mixture of two disaccharides (β-1,6-N-acetylglucosaminyl-N-acetylmuramic acid and β-1,6-N-acetylglucosaminyl-N,4-O-diacetylmuramic acid) and high molecular weight peptides, similar to those obtained on treating the glycopeptide itself with this enzyme. The teichoic acid, the disaccharides and the peptide were separated by gel filtration. This "native" teichoic acid contained no residual components

[4] It has previously been reported that the teichoic acid prepared after 16 hours extraction in 10 % TCA in the cold, contained only 1 phosphomonoester end group per 40 phosphates, compared to the value of 1 phosphomonoester per 14 phosphates in the teichoic acid prepared between 16 and 72 hrs in cold TCA (Sanderson et al., 1962). Still briefer extraction in cold TCA (15 - 30 min) yielded a small amount of a teichoic acid with only 1 phosphomonoester per 120 phosphates. This material, on acid hydrolysis, contained all of the components of the glycopeptide, as did the material prepared after 16 hrs extraction. It is clear, therefore, that small amounts of materials with the properties of the teichoic acid-glycopeptide complex in a mixture with degraded material can be obtained by treatment with 10 % TCA if the period of exposure is sufficiently brief.

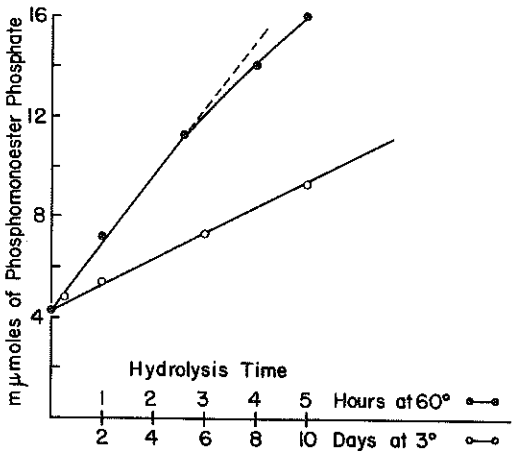

Fig. 1. Hydrolysis of phosphodiester bonds of teichoic acid in 10 % TCA at 3° and at 60°. A preparation of teichoic acid extracted with TCA and containing 1 phosphomonoester end group per 7 phosphates was incubated in 10 % TCA at 3° or 60°. Aliquots containing 29 mμmoles of organic phosphate were removed at intervals. After incubation with E. coli phosphomonoesterase at 38° for 1 hr at pH 7.5, inorganic phosphate was measured.

of the peptide subunit and still contained no phosphomonoester group.[5] Its electrophoretic mobility at the four pHs employed was greater than that of the teichoic acid prepared through the action of TCA.[2]

It has also been demonstrated that phosphodiester bonds are cleaved by TCA under conditions employed to extract teichoic acid from the walls. A rapid increase in phosphomonoester groups occurred at 60° and a slower increase at 3°, thus demonstrating that cleavage of phosphodiester bonds occurred under these conditions (Fig. 1). The molecular weight of the acid-extracted teichoic acid was about 7800 (estimated from $s_{20} = 0.74$ S and $D_{20} = 14 \times 10^{-7}$ cm^2/sec) compared to a value of about 23,000 for the native teichoic acid ($s_{20} = 1.66$ S and $D_{20} = 8 \times 10^{-7}$). These physical measurements are evidence of degradation of teichoic acid during extraction from cell walls by TCA.

[5] A teichoic acid prepared through the action of TCA on the cell walls (containing 1 phosphomonoester per 14 phosphates) was also treated with the acetylmuramidase and the amidase under the same conditions employed to hydrolyze the cell wall and the teichoic acid-glycopeptide complex. Its phosphomonoester group remained intact, and therefore, the native teichoic acid prepared through the action of these enzymes could not have had its phosphomonoester group removed through the action of a phosphatase in these enzymes.

The teichoic acid obtained on solubilization of the cell wall of S. aureus with an acetylmuramidase, therefore, remains associated with a fraction of the glycopeptide from which it cannot be dissociated by electrophoresis at various pHs, by ultracentrifugation or by ion exchange chromatography[6] and it should be regarded as a single molecular species unless it can be demonstrated that the glycopeptide and teichoic acid components of the complex can be separated by means which can be shown not to be hydrolytic. Moreover, since the teichoic acid in the complex is lacking a phosphomonoester end group and since phosphodiester bonds in teichoic acid are demonstrably cleaved under the conditions in which this polymer is solubilized with 10 % TCA, the most plausible interpretation of these data is that the teichoic acid is linked to the glycopeptide through a phosphodiester linkage which is cleaved in TCA.[7] Attempts to identify the group which blocks the phosphomonoester end of the enzymatically prepared teichoic acid are in progress. Large amounts of material will be required, however, since this end group represents at most 1 - 2 % of the weight of the polymer.

The data presented do not, of course, prove that the teichoic acid is linked to the wall through a phosphodiester linkage and the lack of phosphomonoester end groups in the teichoic acid, isolated after enzymatic hydrolysis, may be susceptible of other interpretations (e.g. the phosphomonoester end could be blocked by another substance,

[6] Limited diffusion through the meshwork of the insoluble wall has been advanced as an explanation for the slow extraction of an ionically linked teichoic acid from the wall in cold TCA (Critchley et al., 1962). This argument is not applicable to the non-separability of the soluble teichoic acid-glycopeptide complex.

[7] One possibility is that the teichoic acid is linked by a phosphodiester to a sugar in the polysaccharide. Hydrolysis by the acetylmuramidase, followed by the amidase, would then result in liberation of disaccharides, the peptide and a teichoic acid to which a fragment of the polysaccharide would remain attached (see Mandelstam and Strominger, 1961, Fig. 1A).

such as methanol, or the normal biosynthetic route to teichoic may result in a polymer lacking a phosphomonoester end). The weight of evidence, however, suggests that the teichoic acid of S. aureus is linked to the wall through some covalent bond.[8] In this respect it appears to be similar to the group-specific carbohydrate of Groups A and C Streptococcus hemolyticus (Krause and McCarty, 1961; Krause and McCarty, 1962) or to the polyol phosphate in the wall of Bacillus megaterium (Ghuysen et al., 1962), all of which remain linked to a small fraction of the glycopeptide after hydrolysis of the walls with specific enzymes. It should be noted that, like the group-specific carbohydrates in S. hemolyticus, the teichoic acid is an immunological determinant in the cell wall of S. aureus (Juergens et al., 1960).

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[8] If the two structures were hydrogen bonded, they might be expected to be separable after heating at neutral pH. After heating at 100° for 30 min, no separation could be obtained by electrophoresis. The fact that the glycopeptide moiety of the complex is degraded, i.e. the polysaccharide portion exists in the form of disaccharides linked to the peptide, also argues against a hydrogen bonded structure. Such a structure might be expected to fall apart from extensive degradation of one of its components.