ON THE STRUCTURE OF THE CELL WALL OF STAPHYLOCOCCUS AUREUS (COPENHAGEN)

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Received August 3, 1961

The cell wall of some strains of S. aureus is composed of two major polymers which can be separated by treatment with cold trichloroacetic acid (TCA) viz: the water-insoluble glycopeptide (1), and the water-soluble ribitol phosphate polymer (teichoic acid) (2). The common occurrence of D-alanine in both polymers, as well as in a cell wall precursor, prompted an investigation of the composition and interrelationship of the two polymers in the cell wall of S. aureus (Copenhagen). Furthermore, quantitative data on cell wall composition in this strain (3), together with assumptions which had been made regarding the structure of the glycopeptide, were not compatible with the occurrence of a large amount of D-alanine in the teichoic acid, as had been found in another strain of S. aureus (2).

Highly purified cell walls from S. aureus (Copenhagen)[1] were treated with cold 10% TCA as employed previously for extraction of teichoic acid (2). The half-time for extraction of phosphate from the wall under these conditions was more than forty hours; even after three weeks 5% of the phosphate remained in the insoluble fraction. These data indicate that the teichoic acid is very

* This investigation was carried out during the tenure of a pre-doctoral fellowship from the National Institute of Allergy and Infectious Diseases, USPHS, and has been supported by USPHS grant E-1902 and NSF grant G-7619

[1] The walls were prepared by disruption of the cells with glass beads, differential centrifugation and purification of the cell wall fraction by digestion with trypsin, deoxyribonuclease and ribonuclease at pH 7. Purification of cell walls by pepsin digestion (pH 1-2, 38°, 15 hours), employed by many investigators, was omitted since it was found that about 40% of the teichoic acid was extracted from cell walls under these conditions or in 0.01 N HCl in the absence of pepsin. It should be emphasized that the conditions of pepsin digestion are analogous to TCA extraction and that degraded cell walls are obtained by this procedure.

tightly linked to the glycopeptide, conceivably by covalent bonds which could be cleaved by acid extraction. After 100 mg. of wall were treated for three weeks with cold TCA, the insoluble residue (glycopeptide) was removed by centrifugation and washed. The ribitol phosphate polymer was precipitated from the combined supernatant solution and washings with acetone and ethanol. Quantitative analyses were then carried out on 1. the original cell wall preparation, 2. the insoluble residue (glycopeptide), 3. the ribitol phosphate polymer, and 4. the supernatant solution (from which precipitation of the components of teichoic acid is not complete).

The cell wall contained, per D-glutamic acid residue, 1.98 D-alanine, 1.04 L-alanine, 0.91 L-lysine, 4.71 glycine, 2.72 N-acetylglucosamine, 0.57 N-acetylmuramic acid, and 1.95 ribitol phosphate residues[2]. Upon separation of the polymers with TCA, the insoluble glycopeptide contained, per D-glutamic acid residue, 1.16 D-alanine, 0.98 L-alanine, 0.85 L-lysine, 4.61 glycine, 1.3 N-acetylglucosamine, 0.44 N-acetylmuramic acid, and 0.16 ribitol phosphate residues. The teichoic acid contained, per original 2.0 ribitol phosphate residues, 1.8 N-acetylglucosamine, and 0.7 D-alanine residues.

A uridine nucleotide cell wall precursor from S. aureus (Copenhagen) contains an acetylmuramyl-peptide with the sequence acetylmuramyl·L-ala·D-glu·L-lys·D-ala·D-ala (5). Furthermore, the peptide containing only a single D-alanine residue does not occur as an intermediate in the synthesis of the complete peptide (6). The ratio of D-alanine to D-glutamic acid, 1.1, in the glycopeptide was, therefore, unexpected. There are two prominent explanations (Figure 1). The glycopeptide might contain a mixture of peptides of sequence L-ala·D-glu·L-lys·D-ala·D-ala and L-ala·D-glu·L-lys in approximately equivalent amounts, or in some other sequences and proportions the average of which yields

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[2] These analyses were carried out following hydrolysis at 100° in 6 N HCl for 4 hours and chromatography on a Dowex 50 column. Under these conditions some destruction of amino sugars, especially of muramic acid, takes place. Acetyl has been determined only in the teichoic acid, but other data have shown that the other amino sugar residues are also N-acylated. Ribitol could not be measured quantitatively. During structural studies of the teichoic acid, it has, however, been established that all of the phosphate is present as ribitol phosphate derivatives(4).

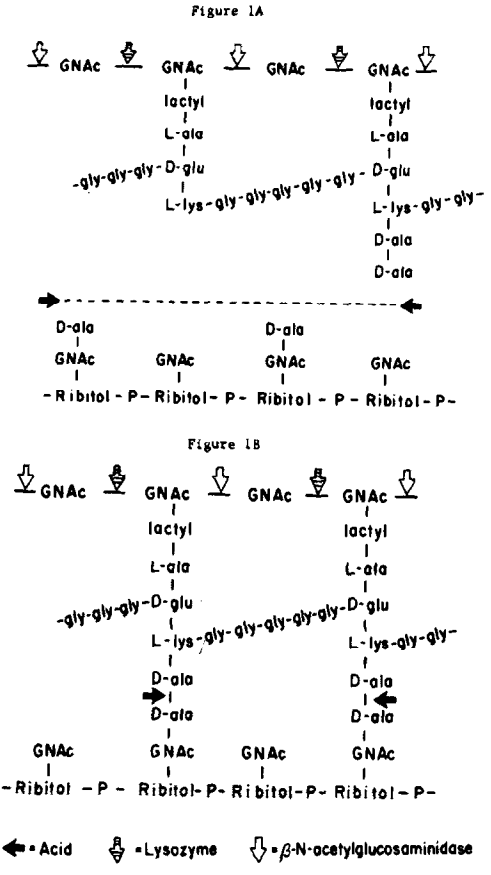

Figure 1. Proposed structure of the cell wall of S. aureus (Copenhagen). In representation A, the linkage between the ribitol phosphate polymer and the glycopeptide is not specified, and it is assumed that the D-alanine residues in these polymers have independent metabolic origins. In representation B, the ribitol phosphate polymer is shown attached to the glycopeptide through the terminal D-alanine residue.

the ratios found (Figure 1A). Alternatively, all of the peptide in the glycopeptide might originally have had the sequence L-ala·D-glu·L-lys·D-ala·D-ala, with the terminal D-alanine esterified to the acetylglucosamine of the ribitol phosphate polymer (Figure 1B). Thus, the two polymers would be linked by a peptide bridge. In this view separation by TCA would be the consequence of peptide bond cleavage and separation of the terminal D-alanine residue of the peptide with the ribitol phosphate polymer.

These possibilities have been examined in several ways. Exhaustive treatment of cell wall with dinitrofluorobenzene (DNFB) yielded only about 0.25 DNP-alanine residue, considerably less than the total alanine residues with free amino groups found in the teichoic acid.[3] However, it remains possible that reaction of the reagent with other alanine residues in the insoluble wall is sterically hindered. It was also unexpectedly found that, after reaction with DNFB, cell wall (or glycopeptide) contained only a trace of DNP-glycine and essentially no α- or ϵ-lysine or other free amino groups which will react with DNFB[4]. In contrast, treatment of the uridine nucleotide cell wall precursor with DNFB yielded a molar equivalent of ϵ-DNP-lysine as a product.

A second approach to the problem is afforded by enzymatic digestion of the glycopeptide, since peptide sequences might be identified in small degradation products, thus distinguishing the two main possibilities under consideration. Several disaccharide-peptide compounds have been obtained from the cell wall of E. coli by this method (7), cf. also (1). Although the wall of S. aureus (Copenhagen) is insensitive to egg white lysozyme, the glycopeptide can be solubilized by this treatment. This solubilization was accompanied by the appearance of a minimum of 0.12 acetylamino sugar residues, reactive as acetylmuramic acid in the modified Morgan-Elson reaction[5] (8) (Figure 2). All the acetylamino sugar remained, however, in a high molecular weight compound. This soluble product contained no additional amino groups reaction with DNFB.

[3] Estimation of DNP-alanine included free-DNP-alanine released during dinitrophenylation as well as that which remained linked to cell wall. No DNP-alanine was found in DNFB-treated glycopeptide.

[4] The amount of DNP-glycine (0.1-0.15 residue per D-glutamic acid residue) from the cell wall and glycopeptide was extremely small. For the present purpose, this amount may be neglected, although it will be discussed in a full publication. The amounts of other free amino groups were less than 0.02 residue. Data obtained by dinitrophenylation of cell wall and glycopeptide have been kindly confirmed by Dr. M.R.J. Salton. Hydrazinolysis carried out by Dr. Salton has also indicated the virtual absence of amino acids with free carboxyl groups in these preparations.

[5] The estimation of appearance of acetylamino sugar groups is based on an acetylglucosamine standard. Free acetylglucosamine and free acetylmuramic acid have nearly the same extention coefficient under the conditions employed. The extinction coefficient of acetylmuramic acid with a free aldehyde group, but held in the polymer by other linkages, may, however, be considerably lower than that of the standard. The determined value would then be lower than the number of acetylmuramic acid residues actually liberated.

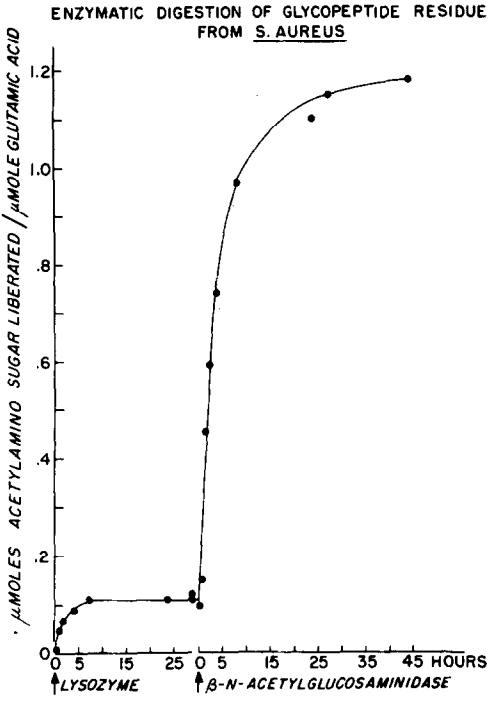

Figure 2 - Appearance of material from the glycopeptide, reactive as acetylamino sugar in the modified Morgan-Elson reaction (8), by consecutive incubation with crystalline egg white lysozyme and with β-N-acetylglucosaminidase from pig epididymis. The rates of hydrolysis are low in this case because extremely small amounts of enzymes were employed to avoid contamination of products with significant amounts of protein. The cell wall itself is not solubilized by either of these enzymes, although β-N-acetylglucosaminidase does liberate some N-acetylamino sugar, presumably from teichoic acid. The glycopeptide is hydrolyzed by β-N-acetylglucosaminidase only after prior treatment with lysozyme.

Further digestion of this soluble glycopeptide with β-N-acetylglucosaminidase from pig epididymis (9) (prepared by A.R. Sanderson) liberated 75-85% of the acetylglucosamine as the monomer (Figure 2). The other products were separated on columns of Sephadex G-25 and G-50 into 3 components. Judging from their behaviors on the columns, two of the components, which contained acetylmuramic acid but virtually no acetylglucosamine, were in the range of molecular weight 3,000-10,000. The third component had a much larger molecular size, contained all of the residual organic phosphate and acetylglucosamine, and about 20% of the acetylmuramic acid with a free aldehyde group; this is further evidence that the organic phosphate is very tightly linked to the glycopeptide. All three components contained the four cell wall amino acids in the same proportion (by semi-quantitative paper chromatographic examination of hydrolysates) and each had a D-alanine to L-alanine ratio near 1. Reaction of each component with DNFB again showed small amounts of DNP-glycine and no other free amino groups.

It is apparent that the glycine is attached covalently to the acetylmuramyl-peptide and it is attractive to postulate that, in addition to the polysaccharide of acetylglucosamine and acetylmuramic acid, the glycopeptide is also polymerized by glycine cross-links (Figure 1). Five glycine residues could provide a bridge between the $\mathcal{E}$-amino group of lysine and a glutamic acid carboxyl group in adjacent peptide chains, thus accounting for the absence of free amino groups and for the large molecular size after removal of acetylglucosamine residues from the polysaccharide polymer. Although the data presented do not conclusively establish this structure, they do restrict the number of possible interpretations. The structures presented in Figure 1 are most in accord with the data available.

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