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STRUCTURE OF LINKAGE REGION BETWEEN GLYCEROL TEICHOIC ACID AND PEPTIDOGLYCAN IN BACILLUS CEREUS AHU 1030 CELL WALLS

Yasuo Sasaki, Yoshio Araki, and Eiji Ito

Department of Chemistry, Faculty of Science,
Hokkaido University, Sapporo 060, Japan

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Summary: Acidic polymer complexes isolated from lysozyme digests of Bacillus cereus AHU 1030 cell walls were found to contain mannosamine and glucosamine in addition to typical components of glycerol teichoic acid and glycopeptide. Mild alkali treatment of the complexes gave a glycopeptide linked to a disaccharide which was identified as N-acetylmannosaminyl(1-4)N-acetylglucosamine, while direct treatment of the cell walls with mild acid yielded the same disaccharide linked to teichoic acid. Thus it was concluded that the glycerol teichoic acid chain in the cell walls is linked to the 6-phosphorylated muramic acid residue of peptidoglycan through this disaccharide unit.

The bacterial wall polymers, such as teichoic acid, teichuronic acid, and other polysaccharides, are believed to be covalently linked to glycan chains of peptidoglycan, but our knowledge of the precise structure of the linkage region between peptidoglycan and the auxiliary wall polymers is still limited. Recently, Baddiley and his collaborators reported that the wall ribitol teichoic acid of Staphylococcus aureus (1) and N-acetylglucosamine 1-phosphate polymer of Micrococcus varians (2) are linked to peptidoglycan through a unique linkage unit composed of a glycerol phosphate trimer and N-acetylglucosamine. In addition, on the basis of the results from biosynthetic (3) and chemical studies (4), the teichuronic acid of Micrococcus lysodeikticus seemed to be attached to peptidoglycan through a saccharide unit containing N-acetylglucosamine as the reducing terminus. The present paper reports the structure of the linkage region between glycerol teichoic acid and peptidoglycan in cell walls of Bacillus cereus AHU 1030.

MATERIALS AND METHODS

Materials and Analytical Methods: Unless otherwise indicated, the materials and methods are the same as those described in the previous paper (5). The

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cell walls of B. cereus AHU 1030 were prepared as described previously (6). N-Acetylmuramoyl-L-alanine amidase was prepared from Flavobacterium L-11 enzyme (7). Sephacryl S-200 was purchased from Pharmacia Fine Chemicals. Total hexosamine was determined by the method of Tsuji et al. (8) after acid hydrolysis of samples (2 M HCl, 100°C, 2 h); glycerol by the enzymatic method (9). Chromic anhydride oxidation was performed as described by Hoffman et al. (10,11) with a small modification. Hexose, N-acetylhexosamine, glycerol, and Smith degradation products were also analyzed, after trimethylsilylation, by Shimazu gas-liquid chromatography on Silicone SE-52 (5%)-coated Chromosorb WAW-DMCS packed in a glass column (3.1 mm x 2 m).

Preparation of Teichoic Acid-Linked Glycan Fragment: The procedures for isolation of teichoic acid-linked glycopeptides were essentially the same as those described in a previous paper (5). N-Acetylated cell walls of B. cereus AHU 1030 (574 mg) were completely digested with an excess amount of lysozyme. The polymer fraction obtained from the lysozyme digests by dialysis and gel filtration on a Sephadex G-50 column was subjected to column chromatography on DEAE-cellulose. An acidic polymer, eluted from the column at about 250 mM $(NH_4)_2CO_3$, was pooled, dialyzed, and subjected to gel filtration on Sephadex G-200 (1.5 x 100 cm) in 50 mM $(NH_4)_2CO_3$. Materials containing hexose and phosphorus emerged as two peaks. The larger (elution volume, 67-86 ml) and smaller acidic polymers (88-120 ml) were separately purified by rechromatography on the same column and denoted as the teichoic acid-linked glycopeptides I (TA-GP-I, 61.8 mg) and II (TA-GP-II, 110 mg).

To prepare a teichoic acid-linked glycan fragment, TA-GP-II (10 mg) was treated with 150 units of N-acetylmuramoyl-L-alanine amidase in 20 ml of 10 mM Tris-HCl, pH 8.2, containing 0.5% NaN_3 at 37°C for 48 h, and then the concentrated reaction mixture was subjected to gel filtration on a Sephacryl S-200 column (1 x 75 cm) in 50 mM $(NH_4)_2CO_3$. Material containing phosphorus and hexose was eluted as a single peak and used as the teichoic acid-linked glycan fragment (TA-G). Reduced TA-G was prepared from TA-G by the $NaBH_4$ treatment.

Mild Alkali Treatment of Reduced Teichoic Acid-Linked Glycan Fragment: The reduced TA-G (28 μmol as phosphorus) was treated in 18 ml of 0.5 M NaOH at 37°C for 30 min. After deionization by passing through a column of Dowex 50 (H^+ form), the hydrolysate was separated into teichoic acid components and a disaccharide-linked glycan fragment by gel filtration on a Sephacryl S-200 column.

Preparation of Disaccharide Component Involved in Linkage Region: The disaccharide-linked glycan fragment (0.4 μmol as phosphorus) obtained as described above was heated in 0.5 ml of 10 mM HCl at 100°C for 30 min. The product was lyophilized, dissolved in 1 ml of 50 mM $(NH_4)_2CO_3$, and subjected to gel filtration on a Sephadex G-25 column (1 x 85 cm) in the same salt solution. The elution profile was monitored by determining phosphorus and total hexosamine. The fractions eluted at the position of the standard chitobiose and the fractions at the void volume were separately pooled and used as the disaccharide fraction and the glycan fragment fraction, respectively.

Preparation of Teichoic Acid-Linked Disaccharide from N-Acetylated Cell Walls: N-Acetylated cell walls (200 mg) were heated in 90 ml of 50 mM glycine-HCl buffer, pH 2.5, at 100°C for 20 min, then cooled and centrifuged at 20,000 x g for 45 min. After dialysis of the resulting supernatant fraction, the nondialyzable fraction was lyophilized (76.7 mg of dry weight). The dried material was dissolved and chromatographed on a Sephacryl S-200 column (1.5 x 100 cm) in 200 mM $(NH_4)_2CO_3$. Fractions containing phosphorus and hexosamine were pooled, dialyzed, and further purified by column chromatography on DEAE-cellulose. Material containing both phosphorus and hexosamine was eluted at about 230 mM $(NH_4)_2CO_3$ and used as the teichoic acid-linked disaccharide fraction after dialysis.

RESULTS AND DISCUSSION

Two different teichoic acid-linked glycopeptide complexes (TA-GP-I and TA-GP-II) were isolated from the lysozyme digests of N-acetylated cell walls of B. cereus AHU 1030 by ion-exchange chromatography and gel chromatography as described in Materials and Methods. Based on the analytical data, the complexes seemed to have an identical teichoic acid moiety made of 60 to 65 repeating glycerol phosphate units which were mostly glucosylated (details are described elsewhere). A teichoic acid-linked glycan fragment (TA-G) prepared by the treatment of the complexes with N-acetylmuramoyl-L-alanine amidase was found to contain muramic acid 6-phosphate, mannosamine, and excess glucosamine in addition to the ordinary components of glycerol teichoic acid and cell wall glycan. Therefore, it seemed possible that mannosamine and glucosamine residues are involved in the linkage region between glycerol teichoic acid and peptidoglycan.

The alkali treatment of TA-G was carried out after reduction with $NaBH_4$ to avoid β-elimination of the lactyl group on the reducing terminal muramic acid residue. The products from alkali treatment of reduced TA-G were separated into two fractions, peaks 1 and 2, by gel filtration on a Sephacryl S-200 column (Fig. 1). The polymer fraction, peak 1, contained phosphorus,

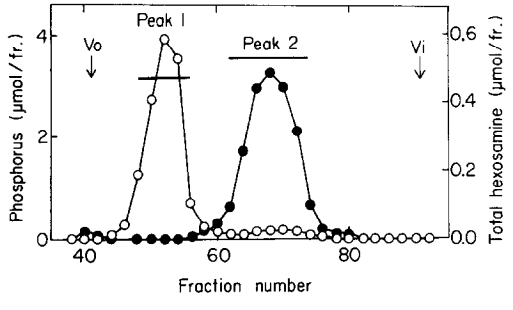

Fig. 1. Chromatography of teichoic acid-linked glycan fragment after mild alkali hydrolysis.
Reduced TA-G (28 μmol as phosphorus) was treated in 0.5 M NaOH at 37°C for 30 min, and the hydrolysate was deionized and chromatographed on a Sephacryl S-200 column in 50 mM $(NH_4)_2CO_3$ as described in Materials and Methods. 0.6-ml fractions were collected and the elution profile was monitored by determining phosphorus (○) and total hexosamine (●). The pooled fractions are indicated in bars.

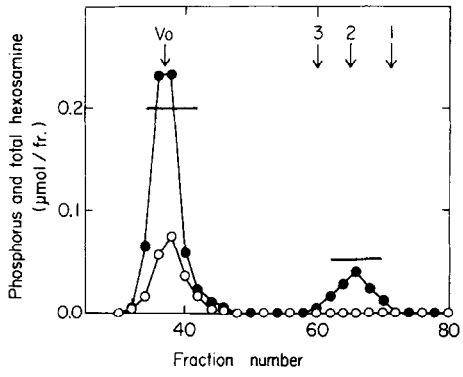

Fig. 2. Chromatography of disaccharide-linked glycan fragment after mild acid hydrolysis.
The disaccharide-linked glycan fragment (0.4 μmol as phosphorus) was treated in 0.5 ml of 10 mM HCl at 100°C for 30 min and lyophilized. The dried material was dissolved in 0.5 ml of 50 mM $(NH_4)_2CO_3$ and chromatographed on a Sephadex G-25 column (1 x 85 cm) in the same salt solution. The symbols used are the same as in Fig. 1. The pooled fractions are indicated in bars. The arrows 1, 2, and 3 indicate the elution positions of the standard saccharides, monomer, dimer, and trimer of N-acetylglucosamine, respectively.

glycerol, and glycose, but it contained only negligible amounts of glycan components. Thus peak 1 seemed to consist of teichoic acid chains. The second fraction, peak 2, contained mannosamine and glycan constituents. The amount of glucosamine exceeded the total of muramic acid derivatives in this fraction. The molar ratio of muramic acid 6-phosphate to mannosamine to excess glucosamine was 1.00:1.16:1.31. When peak 2 was heated in acid (10 mM HCl, 100°C, 30 min), lyophilized, and subjected to gel filtration on a Sephadex G-25 column (Fig. 2), a saccharide was separated from the glycan fragment, which was eluted in the void volumn. The saccharide gave an equimolar amount of glucosamine and mannosamine as analyzed on an amino acid analyzer after acid hydrolysis. When it was hydrolyzed after reduction with $NaBH_4$ and analyzed by gas-liquid chromatography, an equimolar amount of glucosaminitol and mannosamine was given. It contained N-acetyl groups in an amount equivalent to the amino sugars. In the modified Morgan-Elson reaction, the saccharide gave a much lower color yield than N-acetylglucosamine. Smith degradation of the reduced saccharide followed by weak acid hydrolysis yielded an equimolar amount of N-acetylxylosaminitol and glyc-

erol. These results are consistent with N-acetylmannosaminyl(1-4)N-acetylglucosamine for the structure of the saccharide. Based on the data from chromic anhydride oxidation, the mannosamine residue seemed to be of β-anomeric configuration.

The treatment of the glycan fragment with sweet-potato acid phosphomonoesterase (12) resulted in liberation of the phosphoryl group and loss of the muramic acid 6-phosphate residue. In contrast, the phosphomonoesterase treatment had no influence on the original disaccharide-linked glycan fragment. Thus, in the disaccharide-linked glycan fragment, the reducing terminus of the disaccharide moiety seemed to be linked to C-6 of the muramic acid of the glycan fragment through a phosphodiester bridge. The above results strongly suggest that the teichoic acid-linked glycan complex is composed of three structural components, namely, the teichoic acid chain, the disaccharide linkage unit, and the glycan chain, and that the disaccharide is linked to each of the other components through a phosphodiester bridge.

The above suggestion was further supported by the release and isolation of a teichoic acid-linked disaccharide complex from the N-acetylated cell walls by the treatment with 50 mM glycine-HCl buffer, pH 2.5. On the treatment with mild alkali, the complex gave the disaccharide N-acetylmannosaminyl-(1-4)N-acetylglucosamine in addition to teichoic acid chains, and alkaline phosphatase-sensitive phosphoryl groups emerged in parallel with release of the disaccharide from the complex. Analysis of the reducing terminus and Smith degradation studies showed that in the complex the N-acetylmannosamine residue was at the non-reducing terminus and substituted at its 3- or 4-hydroxyl group with the terminal phosphoryl group of the teichoic acid chain. Therefore, it is concluded that the glycerol teichoic acid chain in cell walls is linked to the 6-phosphorylated muramic acid residue of peptidoglycan through a particular disaccharide unit, N-acetylmannosaminyl(1-4)N-acetylglucosamine.

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