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STRUCTURAL AND BIOSYNTHETIC STUDIES ON LINKAGE REGION BETWEEN POLY(GALACTOSYLGLYCEROL PHOSPHATE) AND PEPTIDOGLYCAN IN BACILLUS COAGULANS

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Summary: The HF treatment of teichoic acid-glycopeptide complexes isolated from lysozyme digests of Bacillus coagulans AHU 1366 cell walls gave a disaccharide, glucosylβ(1→4)N-acetylglucosamine, along with dephosphorylated repeating units of the teichoic acid chain, galactosylα(1→2)glycerol. Mild alkali treatment of the complexes yielded the disaccharide linked to glycopeptide, whereas direct heating of the cell walls at pH 2.5 yielded the same disaccharide linked to teichoic acid. The Smith degradation of the complexes revealed that the galactose residue is a component of backbone chain. Thus it is concluded that this disaccharide is involved in the linkage region between poly(galactosylglycerol phosphate) and peptidoglycan in cell walls. Membrane-catalyzed synthesis of this disaccharide on a lipid followed by transfer of glycerol phosphate from CDP-glycerol to the disaccharide-linked lipid in the absence or in the presence of UDP-galactose also supports this conclusion.

The cell walls in each of most strains of Gram-positive bacteria appear to contain one or more kinds of secondary polymers which are covalently linked to peptidoglycan. However, our knowledge of the precise structure of the linkage region between these secondary wall polymers and peptidoglycan is still limited. The following three types of linkage saccharide have been reported: (glycerol phosphate)$_3$-GlcNAc for ribitol teichoic acid and poly-(GlcNAc-1-phosphate) (1); ManNAcUA-GlcNAc for teichuronic acid (2); ManNAc-GlcNAc for glycerol teichoic acid (3). The synthesis of the linkage region in a lipid-linked form is believed to precede the synthesis of the main chains of polymers (1,2,4). On the other hand, the cell walls of several strains of bacilli were found to contain mannosamine in amounts as small as 10 to 35 nmol per mg of cell walls (5), and further studies indicated that the mannosamine-containing disaccharide occurs in most of these strains as a common linkage

Abbreviations used: GlcNAc, N-acetylglucosamine; ManNAcUA, N-acetylmannosaminuronic acid; ManNAc, N-acetylmannosamine.

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unit between glycerol teichoic acids and peptidoglycan (in preparation). However, no mannosamine was detected in the cell walls of some bacteria that appeared to contain glycerol teichoic acid. This fact suggests that another type of saccharide may be present as a linkage unit of the polymer. This paper reports the presence of a novel linkage unit, glucosylβ(1→4)N-acetylglucosamine, which joins poly(galactosylglycerol phosphate) to peptidoglycan in cell walls of Bacillus coagulans AHU 1366.

MATERIALS AND METHODS

Materials and Analytical Methods: Unless otherwise indicated, materials and methods are the same as those described in previous papers (3-6). The cell walls of B. coagulans AHU 1366, kindly supplied by Dr. S. Takao, Hokkaido University, were prepared as described previously (5).

Preparation of Teichoic Acid-Linked Glycan Fragment: Teichoic acid-glycopeptide complexes were isolated from lysozyme digests of N-acetylated cell walls in a procedure similar to that described previously (3). N-Acetylated cell walls (174 mg) were digested with 5 mg of lysozyme at 37°C for 24 h. After dialysis and gel filtration on Sephadex G-50, the resulting polymer fraction was chromatographed on a DEAE-cellulose column. Acidic polymers eluted at about 0.22 M NaCl was separated into two polymer fractions by gel filtration on Sephacryl S-200. The larger and smaller polymers were separately purified by rechromatography on the same column and denoted as TA-GP-I (33.8 mg) and TA-GP-II (72.2 mg), respectively.

To prepare teichoic acid-linked glycan fragments, TA-GP-II (30 mg) was treated with 450 units of N-acetylmuramoyl-L-alanine amidase as described previously (3), then the products were chromatographed on a Sephacryl S-200 column. Material containing both phosphorus and hexosamine was eluted as a single peak and used as the teichoic acid-linked glycan fragment, TA-G.

Preparation of Teichoic Acid-Linked Disaccharide: N-Acetylated cell walls (15 mg) were heated in 3 ml of 25 mM glycine-HCl buffer (pH 2.5) at 100°C for 10 min. After removal of insoluble material by centrifugation, the resulting water-soluble material was dialyzed and chromatographed on a Sephacryl S-200 column. Fractions containing both phosphorus and hexosamine were pooled, dialyzed, and used as the teichoic acid-linked disaccharide.

Mild Alkali Treatment of Teichoic Acid-Linked Glycan Fragment: TA-G was reduced with $NaBH_4$ and the reduction product (38 μmol phosphorus) was treated in 15 ml of 0.5 M NaOH at 37°C for 20 min. After passing through a column of Dowex 50 (H^+), the sample was chromatographed on a Sephacryl S-200 column in 0.05 M $(NH_4)_2CO_3$.

HF Treatment of Teichoic Acid-Glycopeptide Complexes: TA-GP-II (23 μmol phosphorus) was treated in 2 ml of 47% HF at 25°C for 12 h. After removal of HF by evaporation and of anionic material by passing through Dowex 2 (acetate form), the products were chromatographed on a Sephadex G-25 column (1 x 147 cm, superfine) in 0.05 M $(NH_4)_2CO_3$. Material that contained hexosamine and hexose was eluted somewhat behind the position of standard chitobiose and used as the disaccharide fragment (0.75 μmol hexosamine) after purification by rechromatography on the same column. Another material, eluted at the position of GlcNAc as a peak that contained the majority of hexose, was used as the dephosphorylated repeating units of teichoic acid chain (20 μmol hexose) after purification by rechromatography on the same column.

Smith Degradation: TA-GP-II (4 mg) was oxidized with 0.1 M $NaIO_4$ in 0.5 ml of 0.1 M sodium acetate buffer (pH 5.0) for 24 h in the dark at 4°C. After addition of 100 μmol ethylene glycol, the product was reduced with $NaBH_4$ in 0.1 M borate buffer (pH 9.5) and dialyzed. The nondialyzable material was treated in 0.1 M HCl at 25°C for 16 h. After lyophilization, the sample was subjected to gel filtration on Sephadex G-25.

Biosynthetic Studies: The procedures for preparation of membranes from B. coagulans AHU 1366 cells and for incubation of the membranes with labeled nucleotide sugars followed by separation of labeled glycolipids were essentially the same as those described previously (4,6). The conversion of glycerol phosphate-containing lipids was assayed as described previously (4).

RESULTS AND DISCUSSION

Structural investigation on teichoic acid-glycopeptide complexes. From lysozyme digests of N-acetylated cell walls of B. coagulans AHU 1366, two teichoic acid-glycopeptide complexes (TA-GP-I and TA-GP-II) were isolated by ion exchange chromatography and gel filtration as described in Materials and Methods. Both complexes contained glycerol, phosphorus, and galactose in a molar ratio of 1:1:0.95 and also contained small amounts of glycopeptide components and glucose. However, the mannosamine residue present in the cell wall preparation (5) could not be recovered in either complex. For isolation of the linkage unit and dephosphorylated repeating units from teichoic acid-glycopeptide complexes, the complexes were hydrolyzed with HF, and the resulting products were separated by gel filtration. On HF treatment, the teichoic acid-linked glycopeptide obtained from B. cereus AHU 1030 (3) gave a linkage unit disaccharide, ManNAcβ(1→4)GlcNAc, together with dephosphorylated repeating units, glucosylα(1→2)glycerol. Similarly, the treatment of TA-GP-II of B. coagulans AHU 1366 with 47% HF followed by gel filtration on Sephadex G-25 gave a hexosamine-containing disaccharide (peak 1) in addition to a large amount of nonreducing material (peak 2) presumed as dephosphorylated repeating units of the teichoic acid chain (Fig. 1). The hexosamine-containing material in peak 1 gave an equimolar amount of N-acetylglucosamine and glucose as analyzed by gas-liquid chromatography after acid hydrolysis (2 M HCl, 100°C, 2 h) followed by N-acetylation. When it was hydrolyzed after reduction with $NaBH_4$ and analyzed by gas-liquid chromatography, an equimolar amount of glucosaminitol and glucose was given. In the modified Morgan-Elson reaction, it gave a much lower color yield than N-acetylglucosamine (molar color yield relative to N-

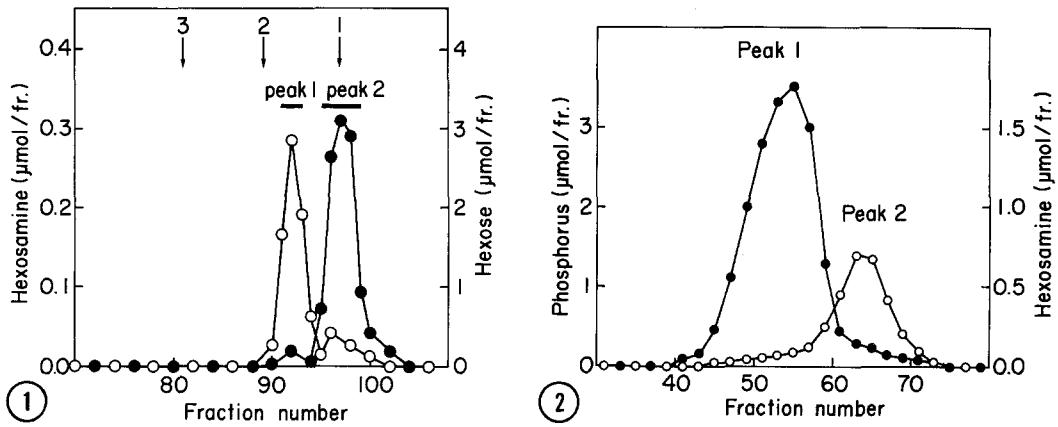

Fig. 1. Isolation of linkage unit and dephosphorylated repeating units from teichoic acid-linked glycopeptide.

TA-GP-II (13 mg, 23 μmol phosphorus) was treated in 47% HF as described in Materials and Methods. After removal of HF and negatively charged materials, the products were chromatographed on a Sephadex G-25 column (1 x 147 cm, superfine) in 0.05 M $(NH_4)_2CO_3$. Fractions (1 ml) were collected and analyzed for hexose (○) and total hexosamine (●). Pooled fractions are indicated in bars. Arrows 1, 2, and 3 indicate the elution positions of monomer, dimer, and trimer of N-acetylglucosamine, respectively.

Fig. 2. Chromatography of teichoic acid-linked glycan fragment after mild alkali hydrolysis.

TA-G was reduced with $NaBH_4$ and the reduction product (38 μmol phosphorus) was treated in 0.5 M NaOH at 37°C for 20 min. The hydrolysate was deionized and chromatographed on a Sephacryl S-200 column (1 x 95 cm) in 0.05 M $(NH_4)_2CO_3$. Fractions (1 ml) were collected and analyzed for phosphorus (○) and total hexosamine (●). Pooled fractions are indicated in bars.

acetylglucosamine, 0.04). In addition, the Smith degradation of reduced saccharide gave N-acetylxylosaminitol. The glucose residue of this saccharide was sensitive to chromic anhydride oxidation. Thus, the most probable structure of this disaccharide is glucosylβ(1→4)N-acetylglucosamine.

On the other hand, nonreducing material in peak 2 gave an equimolar amount of glycerol and galactose on acid hydrolysis. Since the glycerol residue was not degraded by $NaIO_4$ oxidation, the galactose residue was linked to C-2 of the glycerol residue. Furthermore, both TA-GP-I and TA-GP-II gave a signal for anomeric protons ($\delta = 5.180$ ppm; $J_{1,2} = 3.91$ Hz) in 400-MHz proton magnetic resonance spectrum measured in deuteroxide, indicating the α-anomeric configuration of the galactose residue. The Smith degradation of the complexes resulted in complete disappearance of galactose residues with degradation of polymeric chains. On gel filtration of the degradation products through

Sephadex G-25, phosphorus was recovered as a component of a small fragment eluted in front of standard chitobiose. This fragment was shown to contain glycerol and phosphorus in a molar ratio of 2:1. These results indicate that the teichoic acid was composed of repeating units of galactosylα(1→2)glycerol-3(1)-phosphate, which are joined by phosphodiester bonds at C-6 of galactose residues. The apparent molecular weights of the complexes (about 34,000 and 19,000 for TA-GP-I and TA-GP-II, respectively) and the teichoic acid chain (about 14,000) suggest that TA-GP-I and TA-GP-II, respectively, had two and one identical teichoic acid chain made of 30 to 35 repeating units.

A teichoic acid-linked glycan fragment (TA-G) prepared by the treatment of TA-GP-II with N-acetylmuramoyl-L-alanine amidase was found to contain muramic acid 6-phosphate and glucose in addition to the components of teichoic acid and wall glycan. The products from alkali treatment of reduced TA-G were separated into two fractions, peaks 1 and 2, by gel filtration on Sephacryl S-200 (Fig. 2). The polymer fraction (peak 1) purified by rechromatography on the same column contained phosphorus, glycerol, and galactose, but it contained only very small amounts of glycan components. Thus, peak 1 seemed to consist of teichoic acid chains. The second fraction (peak 2) contained glucose 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 glucose to excess glucosamine was 1.0:1.05:1.07. When material in peak 2 was heated in mild acid (0.01 M HCl, 100°C, 30 min), lyophilized, and subjected to gel filtration on Sephadex G-25 (Fig. 3), a saccharide was separated from the glycan fragment, which was excluded from the column. Judging from the analytical data, this saccharide was identical with the disaccharide resulting from HF treatment of the complexes. On the other hand, a compound that contained both teichoic acid and disaccharide was obtained from the N-acetylated cell walls after heating in 25 mM glycine-HCl buffer (pH 2.5) at 100°C for 10 min as described in Materials and Methods. On the treatment with mild alkali, this compound gave the disaccharide glucosylβ(1→4)N-acetylglucosamine in addition to teichoic acid chains. Thus,

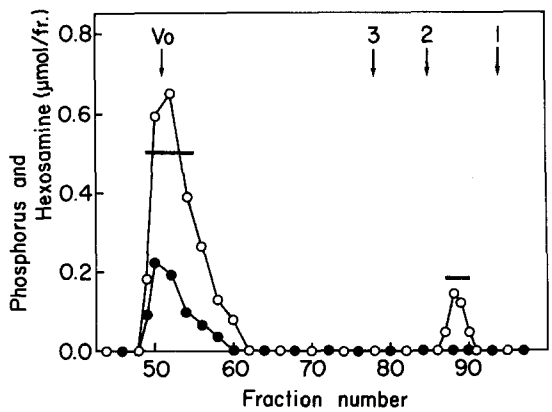

Fig. 3. Chromatography of disaccharide-linked glycan fragment after mild acid hydrolysis.

Hexosamine-containing material (peak 2 in Fig. 2) was purified by rechromatography on the same column, and the disaccharide-linked glycan fragment (2 μmol phosphorus) was treated in 0.2 ml of 0.01 M HCl at 100°C for 30 min and lyophilized. The dried material was dissolved in 0.2 ml of 0.05 M $(NH_4)_2CO_3$ and chromatographed on a Sephadex G-25 column (1 x 147 cm, superfine) in the same salt solution. Fractions (1 ml) were collected and analyzed for phosphorus (○) and total hexosamine (●). Pooled fractions are indicated in bars. Arrows 1, 2, and 3 indicate the elution positions of monomer, dimer, and trimer of N-acetylglucosamine, respectively.

the teichoic acid and glycan chains seem to be attached to the nonreducing and reducing ends, respectively, of the linkage disaccharide unit in the teichoic acid-glycopeptide complexes.

The above results lead to a conclusion that the poly(galactosylglycerol phosphate) chain in the cell walls of this strain is linked to peptidoglycan through a novel linkage unit, glucosylβ(1→4)N-acetylglucosamine.

Biosynthetic studies. When the membranes from this strain were incubated with UDP-[^{14}C]GlcNAc, a GlcNAc-linked lipid presumed as N-acetylglucosaminyl pyrophosphorylprenol was yielded. In addition, incubation of the membranes with the GlcNAc-linked lipid and UDP-[^{14}C]glucose led to formation of a disaccharide-linked lipid, of which the saccharide moiety was tentatively characterized as glucosyl-GlcNAc on the basis of analytical data. On paper chromatography in several solvents, the saccharide moiety from the disaccharide-linked lipid was coincident with the disaccharide unit obtained from HF treatment of TA-GP-II. Furthermore, the disaccharide-linked lipid functioned as an acceptor of glycerol phosphate units from CDP-glycerol with formation

of a series of glycerol phosphate-containing lipids. On gel filtration, mild acid hydrolysates of the synthesized glycerol phosphate-containing lipids gave three or more peaks of radioactive materials. The radioactive materials seemed to possess one, two, three or more negatively charged groups based on their electrophoretic mobility. Thus, mono-, di-, and tri(glycerol phosphate) linked to disaccharide-lipid were formed in this membrane system. The membranes also catalyzed the incorporation of radioactivity from the labeled disaccharide-linked lipid and CDP-[^{14}C]glycerol into the polymer fraction in the presence of UDP-galactose. Therefore, it seems most probable that the biosynthesis of the wall teichoic acid in this strain involves the disaccharide-linked lipid as an intermediate. This result also supports the structure of the linkage unit between poly(galactosylglycerol phosphate) and peptidoglycan mentioned above.

The present and previous (3) studies on the linkage regions strongly suggest that the wall glycerol teichoic acid chains in various strains are linked to peptidoglycan through either of the two different types of linkage saccharides, glucosyl-GlcNAc and ManNAc-GlcNAc. It is of great interest to know whether this structural difference in the linkage saccharides is related to the structural difference in the backbone chains of teichoic acids or related to bacterial species. Further studies on the detail enzymatic process of poly(galactosylglycerol phosphate) synthesis in B. coagulans AHU 1366 are in progress.

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