β-D-Glucose 1-Phosphate

A STRUCTURAL UNIT AND AN IMMUNOLOGICAL DETERMINANT OF A GLYCAN FROM STREPTOCOCCAL CELL WALLS

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Glycose 1-phosphate moieties are emerging as important structural units of macromolecular substances imparting special biological functions to these molecules. In the present study, β-D-glucose 1-phosphate moieties are shown to be structural units and immunological determinants of a bacterial glycan. The glycan is a tetraheteroglycan from the cell wall of Streptococcus faecalis, strain N and is composed of glucose, rhamnose, N-acetylgalactosamine, and phosphate. Several lines of evidence have been obtained for the presence of β-D-glucose 1-phosphate units in the glycan, including the liberation of glucose by mild acid hydrolysis, the inhibition of the precipitin reaction by β-D-glucose 1-phosphate, and the formation of levoglucosan on treatment of the glycan with alkali. Work on the preparation of affinity adsorbents for isolating the new types of antibodies directed at the β-D-glucose 1-phosphate moieties is in progress.

It was reported earlier that terminally linked glycose 1-phosphate units were structural units of immunogenic glycans in the cell wall of some strains of bacteria and yeasts (1–4). These units were glucose 1-phosphate, N-acetylglycosamine-1-phosphate and mannose 1-phosphate and were shown to be joined by phosphate diester bonds to other units of the glycans. In the naïve glycans, these units functioned as immunological determinants. Subsequently, glucose 1-phosphate was identified as a structural unit of dolichyl-glycosyl-phosphate functioning as a donor of glucose units in the enzymatic synthesis of glycoproteins (5, 6). More recently, N-acetylglycosamine-1-phosphate moieties have been found to be structural units of some glycoproteins in which the unites function as regulators for the transport of glycoproteins through cellular membranes (7, 8).

The configuration of the hexose units in the glycos 1-phosphates is, for the most part, α. The configuration of the glucose moieties of the immunogenic tetraheteroglycan from Streptococcus faecalis has now been shown to be β. The β-D-glucose 1-phosphate units are rare in biological molecules and are of further interest because of their immunological properties. The other carbohydrate units of the tetraheteroglycan are rhamnose, galactose, and N-acetylgalactosamine (9). The cell wall of S. faecalis contains another immunological glycan, a diheteroglycan of glucose and galactose for which structural and immunological properties have been recorded (10).

Methylation data on the tetraheteroglycan have been obtained showing that the glucose 1-phosphate moieties are attached as terminal side chains to α-mannose units of the glycan. Immunological studies have shown that antibodies directed against the β-D-glucose 1-phosphate units of the glycan were present in the sera of rabbits immunized with a vaccine of nonviable cells of the organism. The antibodies have been especially useful for investigating structural features of the glycan of importance in the antigen-antibody reaction.

EXPERIMENTAL PROCEDURES

The procedures for the isolation and the purification of the glycans of the wall of S. faecalis, strain N have been described (9). Two immunogenic glycans, a diheteroglycan of glucose and galactose and a tetraheteroglycan of rhamnose, glucose, galactose, and N-acetylgalactosamine, are present in the cell wall of this organism. The tetraheteroglycan also contains a small amount of phosphate. Methylation of the glycans were performed following the methods described in the literature (12, 13). The data for the diheteroglycan have been published (14), but the data for the tetraheteroglycan are incomplete. In the present study, 2-mg samples of the tetraheteroglycan and suitable reference compounds were subjected to methylation and to analysis by gas-liquid chromatography and mass spectrometry (12, 13).

The following methyl alditol acetates, with retention times and molar ratios in parentheses, were identified in the reaction mixture of the tetraheteroglycan: 1,2,5-tri-O-acetyl-3,4-di-O-methyl rhamnitol (0.86, 2 mol), 1,3,5-tri-O-acetyl-2,4-di-O-methyl rhamnitol (0.91, 4 mol), 1,6-di-O-acetyl-3,4,6-tetra-O-methyl glucitol (1.00, 4 mol), 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl galactitol (2.22, 1 mol), and 1,5,6,2-tetra-O-acetyl-2,4-di-O-methyl galactitol (4.95, 1 mol). The mass spectra of these derivatives were identical with those of authentic samples (12). The derivatives of N-acetylgalactosamine have not yet been identified. The methylation data reveal two important structural features of the glycan. First, the glucose units are terminal units and are present as side chains on a main chain of the glycan. Second, the main chain is composed of rhamnose, galactose, and N-acetylgalactosamine since galactose oxidase test strips (15) showed that the galactose and the N-acetylgalactosamine moieties were not terminal units.

Antisera obtained by immunization of rabbits with nonviable cells of the organism contain antibodies directed at both the tetraheteroglycan and the diheteroglycan. Agar diffusion patterns with the antisera and the glycans are shown in Fig. 1. Quite clearly, the two glycans are different antigens as the precipitin bands were present at different locations on the plate. Agar diffusion was also performed with solutions of the glycan which had been subjected to mild acid treatment. Samples of 1 mg of the glycans were dissolved in 0.02 ml of 0.01 n HCl and heated in sealed tubes at 100 °C for 1 h. The neutralized samples were then used in agar diffusion tests and the results are shown in Fig. 1. It will be noted in the figure that the acid treatment had no effect on the antigenicity of the diheteroglycan.

However, heating the tetraheteroglycan under the above conditions yielded a product which did not give a precipitin band in agar diffusion tests. In the hydrolysate of the tetraheteroglycan, glucose was identified by paper chromatography in the solvent system, n-butyl alcohol-pyridine-water, 6:4:3 by volume and by glucose oxidase test strips (16). The reducing compound in the hydrolysate and reference glucose possessed identical RF values of 0.41. Both compounds also yielded positive tests with glucose oxidase. Samples of α- and β-D-glucose 1-phosphate were also subjected to acid treatment under the above conditions and to paper chromatographic analysis. The rates of glucose production from these compounds were compared to the rate of glucose production from the tetraheteroglycan.

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In order to identify the hapten group of the glycan, glucose, rhamnose, galactose, N-acetylgalactosamine, and several glucose derivatives were tested in quantitative hapten inhibition tests as described earlier (9). In these tests an optimal level of antigen (80 μg), a constant level of antiserum (0.1 ml), and varying amounts of inhibitor (0.04 to 0.2 μm) in a final volume of 1 ml were incubated overnight at 4°C. The precipitates which formed were collected and quantitatively determined (9). The control samples without inhibitor yielded an absorbance value of 0.7 and without antigen and inhibitor a value of 0.02. Plots of the data for some of the inhibition tests are shown in Fig. 2. It is apparent that only β-glucose 1-phosphate was an inhibitor of the precipitin reaction. All other compounds which were tested exhibited low levels of inhibition. The immunological determinant of the tetraheteroglycan is β-D-glucose 1-phosphate.

A sample of 50 mg of the tetraheteroglycan was dissolved in 5 ml of water, adjusted to pH 8.0 with 0.1 N NaOH, and stirred with 40 mg of sodium borohydride. The reaction mixture was allowed to stand at room temperature for 24 h, then was acidified to pH 4.5 with acetic acid and dialyzed against distilled water for 48 h. The reduced glycan was taken to dryness by lyophilization, and the yield was 40 mg. A sample of 4 mg of reduced tetraheteroglycan was dissolved in 0.1 ml of 0.1 N NaOH in a glass vial. The vial was sealed and then heated at 115°C for 6 h. It has been shown earlier (6) that under these conditions β-glucosides but not α-glucosides yield levoglucosan. At the end of the heating period, the vial was opened and the solvent evaporated under vacuum. The reaction products were then acetylated in 0.2 ml of dry acetic anhydride and 0.2 ml of pyridine at room temperature for 6 h. The reagents were removed by evaporation in a stream of nitrogen and the products were dissolved in a small volume of dry chloroform. This solution was subjected to gas-liquid chromatography analysis. Gas-liquid chromatographic patterns for some of the samples are shown in Fig. 3. It will be noted that the reduced tetraheteroglycan when heated in dilute alkali yielded a product which on acetylation possessed a retention time on gas-liquid chromatographic analysis identical with that of authentic 2,3,4-tri-O-acetyl levoglucosan. A sample of the reduced tetraheteroglycan which was not treated with alkali did not yield this product. Also β-D-glucose 1-phosphate when subjected to the above series of reactions yielded the levoglucosan derivative but α-D-glucose 1-phosphate did not.

RESULTS AND DISCUSSION

Fig. 1 shows agar diffusion plates in which the tetraheteroglycan (well 2) yielded an intense precipitin band with the homologous antiserum (well S). The figure also shows that the diheteroglycan from S. faecalis (well 1) yielded an intense precipitin band. Since the precipitin bands obtained with a mixture of glycans (well 3) and the antiserum (well S) were located at different positions in the agar, the glycans are two different antigens. The minor degree of fusion of the tetraheteroglycan band (well 2) with the diheteroglycan band (well 1) noticeable in Fig. 1 was not apparent in several other diffusion tests performed at different concentrations of antigens and antiserum. A sample of the tetraheteroglycan which was heated in dilute acid did not yield a precipitin band (well 5), but a sample of the diheteroglycan treated in the same manner did (well 4). The latter results are also shown in Fig. 1. Quite clearly, the tetraheteroglycan contains acid-labile bonds which on hydrolysis yield a product which is no longer antigenic. Glucose was identified in the hydrolysate of the tetraheteroglycan by paper chromatography and by glucose oxidase. The release of glucose from the tetraheteroglycan and from α- or β-D-glucose 1-phosphates occurred at the same rate.

Quantitative hapten inhibition tests were performed with glucose, rhamnose, galactose, N-acetylgalactosamine, and a number of glucosyl compounds. Results of some of these experiments are shown in Fig. 2. It can be seen in the figure that the precipitin reaction was inhibited strongly by β-D-glucose 1-phosphate but not by the other compounds tested. The β-D-glucose 1-phosphate is, therefore, the immunodeterminant group of the tetraheteroglycan.

Samples of the reduced tetraheteroglycan and appropriate
standards were heated in dilute alkali and the reaction products were analyzed by acetylation and gas-liquid chromatography. The patterns for three samples are shown in Fig. 3. It is noted in the figure that the tetraheteroglycan yielded a product which had the same retention time as authentic 2,3,4-tri-O-acetyl levoglucosan. This product was not present in a control of the glycan. In other experiments, it was found that β-D-glucose 1-phosphate yielded levoglucosan, but α-D-glucose 1-phosphate did not when subjected to the same series of reactions. These findings substantiate further the conclusion that the tetraheteroglycan contains β-D-glucose 1-phosphates as structural units.

Carbohydrate antigens with different types of immunodeterminants are present in the cell walls of many groups and strains of Streptococci (11). Such immunodeterminants form the basis for the scheme for the serological classification of these organisms. In the immune response, these determinants combine with the receptor substances on the immunocytes to form a complex. The complex initiates the series of reactions which lead to the synthesis of anti-glycosyl antibodies. The β-D-glucose 1-phosphate unit is not a common structural feature of biological molecules and most likely imparts unusual properties to such molecules. The presence of β-D-glucose 1-phosphate units in the tetraheteroglycan from the cell wall of S. faecalis, strain N and the occurrence of antibodies directed against such units has not been observed previously.

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J H Pazur


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