The Structure of a Polymer containing Galactosamine from Walls of *Bacillus subtilis* 168

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Cell walls of *Bacillus subtilis* 168 contain a phosphorylated polysaccharide composed of *N*-acetylgalactosamine, glucose and phosphate. Controlled acid hydrolysis gives 3-*O*- β -D-glucopyranosyl-*N*-acetylgalactosamine with a phosphomonoester group at the 6-position of glucose. It is likely that the polysaccharide consists of disaccharide units connected by phosphodiester residues joining the 1-position of *N*-acetylgalactosamine to the 6-position of glucose in the neighbouring unit.

We have shown (Duckworth *et al.*, 1972) that walls of *Bacillus subtilis* 168 contain *N*-acetylgalactosamine in two distinct polymers. One of these is extracted when walls are treated with dilute NaOH and can be separated from the glycerol teichoic acid by ion-exchange chromatography. The purified material contains *N*-acetylgalactosamine, glucose and phosphate and is labile in dilute acid under conditions that hydrolysed sugar 1-phosphate linkages. Hydrolysis under these conditions gives glucose 6-phosphate and a phosphate of glucosyl-*N*-acetylgalactosamine. The work described in the present paper establishes that the disaccharide phosphate is 2-acetamido-2-deoxy-3-*O*-(6-*O*-phosphoryl- β -D-glucopyranosyl)-D-galactose.

Two procedures were used for the large-scale preparation of disaccharide phosphate. Treatment of walls with 0.1 M-HCl at 100°C for 10 min gave a mixture of monosaccharide phosphate and disaccharide phosphate. Some preparations of walls gave approximately equal amounts of the two phosphates whereas others gave only very small amounts of the monosaccharide phosphate. The two phosphates were separated by preparative paper electrophoresis, which gave disaccharide phosphate that was free of glucose phosphate but contaminated by small amounts of P_i and glucosylglycerol phosphate, the latter presumably being derived by partial degradation of the glycerol teichoic acid. Subsequently, pure preparations of disaccharide phosphate were obtained as follows. Walls were treated with 0.1 M-HCl at 37°C for 16 h. Acid was removed from the extract by evaporation *in vacuo* over KOH pellets, and an aqueous solution (10 ml) of the material (171 μ mol of phosphate) was then applied to a column (190 mm $\times$ 10 mm) of Dowex 1 (formate form) resin. Material was eluted with a linear gradient of 0–2.4 M-formic acid (1500 ml) at 30 ml/h. Two peaks representing phosphomonoesters were observed, and fractions corresponding to the first of these yielded pure disaccharide phosphate

(5.75 μ mol). Traces of formic acid were removed by twofold evaporation with ethanol followed by drying over KOH pellets *in vacuo*. The disaccharide phosphate contained equimolar amounts of *N*-acetylgalactosamine, glucose and phosphate. Incubation with calf intestinal phosphomonoesterase in 0.1 M-ammonium carbonate, pH 9, gave a single organic product that, after reduction with NaBH₄ and hydrolysis in 2 M-HCl at 100°C for 3 h, gave glucose and galactosaminitol. Hydrolysis of the disaccharide phosphate with 3 M-HCl at 100°C for 3 h gave glucose phosphate and galactosamine together with a minor component containing phosphate; this minor component was positively charged at pH 5.3 and had R_{Glc} 0.27 on chromatography in pyridine–ethyl acetate–acetic acid–water (5:5:1:3, by vol.). These properties suggest that this minor component is deacylated disaccharide phosphate. The glucose phosphate reacted with glucose 6-phosphate dehydrogenase; however, this product was formed under conditions that would cause phosphate migration and so this evidence does not indicate the original location of the phosphate. The observations do, however, establish that the disaccharide phosphate is a glucosyl-*N*-acetylgalactosamine with a phosphomonoester group attached to one of the free hydroxyl groups of glucose.

On reaction with the Morgan–Elson reagents (Reissig *et al.*, 1955) the disaccharide phosphate gave a reaction corresponding to 2–3 times the equivalent amount of *N*-acetylgalactosamine. This is characteristic of 3-substituted acetylhexosamines (Kuhn *et al.*, 1954). The position of the glycosidic linkage was confirmed by periodate oxidation of a sample of disaccharide phosphate that had been reduced with NaBH₄. The reduced disaccharide phosphate reduced 4 mol. prop. of periodate (Avigad, 1969) and yielded 1 mol. prop. of formaldehyde (Hanahan & Olley, 1958). These results are consistent only with a structure in which glucopyranose is attached to the 3-position of *N*-acetylgalactosaminitol and the phosphate is

Fig. 1. Structure of the disaccharide phosphate and its probable linkage in the phosphorylated polysaccharide

esterified to the 6-position of the glucose. The disaccharide phosphate, the disaccharide and the reduced disaccharide were not hydrolysed by incubation in aqueous solution with yeast α -glucosidase or emulsin β -glucosidase, presumably because of the nature of the aglycone, but the disaccharide phosphate had a low positive optical rotation, suggesting that the anomeric linkage has the β -configuration. Optical-rotatory-dispersion studies showed that the molar rotation of the disaccharide phosphate is much lower than that of *N*-acetylgalactosamine and that the first Cotton effect is negative. This has been found only for β -linked disaccharides (Englard *et al.*, 1966), and we conclude that the glucopyranosyl linkage has the β -configuration. This conclusion is based on the assumption that the phosphate group does not substantially affect the optical rotation of the sugar; this assumption is reasonable since the molar rotation of glucose, $[\phi] = 95^\circ$, does not differ greatly from that of glucose 6-phosphate, $[\phi] = 71^\circ$.

The properties of the isolated disaccharide corresponded closely to those described for the identical disaccharide isolated from reduced chondroitin (Wolf from & Juliano, 1960), from cell-wall polysaccharides of *Lactobacillus casei* (Knox & Hall, 1965) and from group F streptococci (Michel & Willers, 1964). The reduced disaccharide has also been described (Davidson & Meyer, 1954).

The phosphorylated polysaccharide thus contains repeating structures of 2-acetamido-2-deoxy-3-*O*-(6-*O*-phosphoryl- β -D-galactopyranosyl)-D-galactose. The polysaccharide can be extracted from walls by treatment with dilute alkali and can then be separated from glycerol teichoic acid by ion-exchange chromatography (Duckworth *et al.*, 1972). Selective extraction of the polysaccharide can be achieved by treatment of walls with 0.1 M-citrate or acetate buffer at pH 4.0 for 20 min at 100°C (Pavlik & Rogers, 1973). The lability of the polymer in dilute acid indicates that units are joined together by sugar 1-phosphate linkages, the probable structure of the polysaccharide being that shown in Fig. 1. It should be noted that,

although shown in the α -configuration, there is no direct evidence for the anomeric configuration of the *N*-acetylgalactosaminyl 1-phosphate linkage.

Controlled acid hydrolysis of walls also gives glucose 6-phosphate. It is unlikely that this is derived by hydrolysis of the disaccharide phosphate since this would give equivalent amounts of *N*-acetylgalactosamine or galactosamine, neither of which was found. It is possible that glucose 6-phosphate units are present in the same polymer as are the disaccharide phosphate units, but a regular structure is unlikely since greatly differing proportions of glucose phosphate and disaccharide phosphate are produced from different batches of wall. Possibly the glucose 6-phosphate is the repeating unit of a separate polymer that is present in variable amounts in the wall. Further work would be required to establish this, but we do not plan to continue this investigation.

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