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Proposed Pathway for Biosynthesis of the S-Layer Glycoprotein of *Bacillus alvei*

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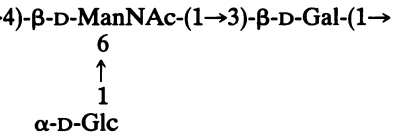
The outer surface of the murein sacculus of the eubacterium *Bacillus alvei* is covered by a surface layer (S-layer) glycoprotein. The glycan chain of this S-layer glycoprotein consists of trisaccharide repeating units with ManNAc, Gal, and Glc as constituents. From cell extracts of *B. alvei*, nucleotide-activated derivatives of ManNAc, Gal, Glc, and GlcNAc were isolated. Furthermore, GDP- and dolichyl-activated oligosaccharides were obtained. On the basis of the isolated putative glycoprotein precursors, a pathway for the biosynthesis of the oligosaccharide chain is proposed.

Glycosylated proteins are common in eukaryotes and prokaryotes. In prokaryotes, glycoproteins have been found mainly as structural elements of the cell envelope (for reviews, see references 11 and 16). The outer cell surface of the gram-positive, mesophilic eubacterium *Bacillus alvei* is covered by an S-layer glycoprotein. The trisaccharide repeating unit



was characterized as a building block of the glycan (3). So far, the biosynthesis of only two archaebacterial S-layer glycoproteins has been studied in detail (8, 14, 19, 23). Both the chemical structure and the biosynthesis of these archaebacterial glycoproteins show large differences in comparison with eukaryotic glycoproteins. Here we report on the biosynthesis of a eubacterial S-layer glycoprotein.

MATERIALS AND METHODS

Organism. *B. alvei* (CCM 2051) was from the Universität für Bodenkultur, Wien, Austria. The cells were grown in a 10-liter fermenter in LBO medium (10.0 g of tryptone, 5.0 g of yeast extract, 5.0 g of NaCl, 1,000 ml of double-distilled H_2O [pH 7.2]).

Preparation of cell extracts. The preparation of cell extracts with aqueous trichloroacetic acid (100 g/liter, 80 ml/50 g of wet cells, 0°C [12]) or sodium acetate buffer (0.1 M, pH 4.2)–1-butanol (1:2 [9]) was performed as described previously.

Chromatography. Thin-layer chromatography (TLC) was performed by using the following TLC plates and solvents (by volume). For polyethyleneimine-cellulose (Merck), solvent a was 0.2 M triethylamine-CO_2 buffer (pH 8.0) (running distance, 16 cm) and solvent b was 1 M LiCl in 0.01 M triethylamine-CO_2 buffer (pH 8.0) (running distance, 10 cm). For cellulose F254 (aluminum sheets; Merck), solvent c was isobutyric acid-ammonia (25%)-water (198:6:99) (running distance, 16 cm). For Silica Gel 60 F254 (aluminum sheets; Merck), solvent d was butanol-ethanol-water (5:3:2) (running distance, 10 cm), solvent e was chloroform-methanol-water (60:25:4) (running distance, 16 cm), and solvent f was isopropanol-water (7:3) (running distance, 12 cm). Bands containing nucleotides were detected in UV light (254 nm). Stain reagents were anisidine phthalate (sugars), ninhydrin (amino sugars), and iodine vapor (lipid).

Column chromatography. Gel filtration (8) was performed on a Sephadex G-25 fine column (2 by 120 cm; Pharmacia). The eluent system consisted of 0.02 M triethylamine-CO_2 buffer (pH 8.0). The elution profile was recorded at 260 nm. Flow rate was 2 ml/min.

Analytical methods. Amino sugars were determined with a Biotronic LC5000 amino acid analyzer. Neutral sugars and amino sugars were identified as alditol acetates (1) by gas-liquid chromatography (Hewlett-Packard model 5880A) on a Durabond 1701 capillary column (25 m; ICT Laboratories). The reducing sugars of the oligosaccharides were determined as the corresponding alditol derivatives after reduction with $NaBH_4$; in the case of the activated intermediates, the reduction was performed after splitting off the nucleotide diphosphate (hydrolytic condition e) or lipid pyrophosphate residue (hydrolytic condition d). Quantitative determination of neutral sugars was performed enzymatically (5). Periodate oxidation was carried out with 0.1 M $NaIO_4$ in 0.1 M sodium acetate buffer (pH 5) for 16 h at 4°C. The different bases were identified and quantified as described previously (7, 12) by determination of the absorbance ratios and cochromatography with standards after treatment with phosphodiesterase. Quantitative determination of phosphate was performed by the molybdate method (6). Phosphodiester bonds were determined with phosphodiesterase (13). N acetylation of the amino sugars was determined by the direct Morgan-Elson test after mild acid hydrolysis (18). N acetylation of the amino sugars was carried out with acetic acid anhydride (22).

Mass spectrometry. Mass spectrometric measurements were carried out by using a linear californium-252 plasma desorption time-of-flight mass spectrometer (Applied Biosystems, Foster City, Calif.) with a flight tube length of 150 mm. The acceleration voltage was 17 kV in the positive ionization mode and 15 kV in the negative ionization mode. Spectra measured in the positive and negative ionization

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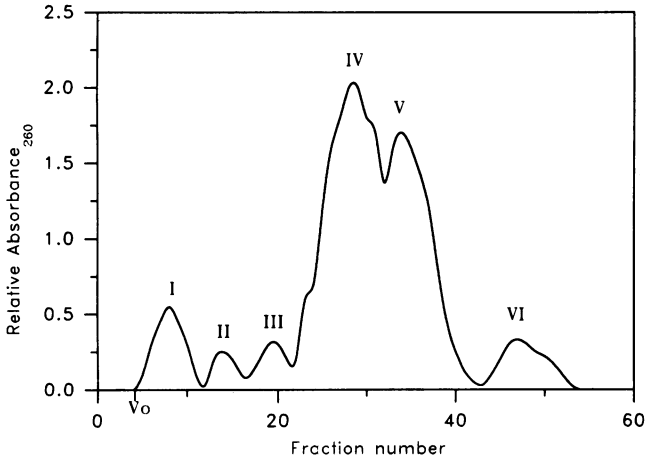

FIG. 1. Elution profile of trichloroacetic acid-extracted cells of *B. alvei* separated on a Sephadex G-25 fine column. I to VI indicate peaks obtained; void volume is 90 ml.

modes were accumulated for 5 million and 10 million fission events, respectively.

Mass calibration was based on H^+ and on the $[M+H]^+$ ion of the matrix 3-(3-pyridyl) acrylic acid (PAA) (*m/z* 150,0555, monoisotopic) in the positive ionization mode and on H^- and the $[M-H]^-$ ion of the matrix (*m/z* 148,0398, monoisotopic) in the negative ionization mode.

PAA surfaces for sample adsorption in plasma desorption mass spectrometry were prepared by electrospraying as described elsewhere (2). The sample was dissolved in 8 μl of *n*-hexane and was spin deposited (17) on a PAA-covered target foil.

Hydrolytic conditions. Hydrolytic conditions were as follows: condition a (amino sugars), 4 M HCl, 16 h, 100°C; condition b (neutral sugars), 2 M HCl, 2 h, 100°C; and condition c (mild acid hydrolysis [lipid pyrophosphates] [8, 15]), 1 drop of 1-propanol in 0.01 M HCl, 5 min, 100°C. For sugar-1-phosphates, conditions were 0.01 M HCl, 30 min, 100°C (condition d) and 0.01 M HCl, 10 min, 100°C (condition e). Condition f (4 M HCl, 30 min, 100°C) was used for partial hydrolysis. Condition g (chloroform–methanol–10 M NaOH [10:10:3 by volume]) was used for dolichyl monophosphate (21). The lipids were extracted from the hydrolysates (conditions c and g) with chloroform.

Standards. Dolichol-C_{80-105}, dolichol-C_{55}, dolichyl-C_{80-105} monophosphate, dolichyl-C_{55} monophosphate, and undecaprenol were obtained from Sigma, Deisenhofen, Germany. GlcNAc, Gal, and Glc were obtained from Serva, Heidelberg, Germany. ManN was obtained from Fluka, Neu Ulm, Germany. PAA was obtained from Aldrich, Steinheim, Germany.

RESULTS

Isolation of the nucleotide-activated precursors. Cells were extracted with aqueous trichloroacetic acid (12), and the extracts were separated on a Sephadex G-25 column (8). The elution profile showed six distinct peaks (Fig. 1). Peaks I and II containing ManNAc or GlcNAc were further separated on cellulose F254 aluminum sheets (solvent c; Table 1). The UV-absorbing bands were eluted and finally purified on polyethyleneimine-cellulose plates (solvents a and b; Table 1).

Characterization of nucleotide-activated monomers. Four nucleotide-activated monomers could be purified from peak II (compounds 3 to 6) by TLC (Table 1). Two compounds consisted of uracil, phosphate, and Glc (compound 3) or Gal (compound 4). Compound 5 was composed of ManNAc, guanine, and phosphate; compound 6 was composed of GlcNAc, uracil, and phosphate. N acetylation of the amino sugars was indicated by a positive direct Morgan-Elson test after removal of UDP (hydrolytic condition e) and a negative ninhydrin reaction. The data (Table 1) indicate that the four compounds are nucleotide-activated sugar monomers.

Characterization of nucleotide-activated oligosaccharides. Two nucleotide-activated oligosaccharides (compounds 1 and 2; Table 1) could be isolated by TLC from peak I (Fig. 1). Compound 1 was composed of guanine, phosphate, ManNAc, GlcNAc, and Gal. Compound 2 additionally contained Glc. Both compounds did not react with ninhydrin. This finding indicates that ManN and GlcN should be N acetylated. The molar ratio of the components (Table 1) indicates that compound 1 is a GDP-activated trisaccharide and compound 2 is a GDP-activated tetrasaccharide (Table 1). Both compounds have ManNAc at the reducing end (see Materials and Methods). After partial hydrolysis, two disaccharides (compounds 1a and 1b) and three disaccharides (compounds 2a, 2b, and 2c) could be isolated by TLC from compounds 1 and 2, respectively (solvent d; Table 2). The molar ratios of the components of compounds 1 and 2 (Table 1), the isolated oligosaccharides (Table 2), structural analysis, and chromatographic behavior indicate that compound 1 is a GDP-activated trisaccharide and that compound 2 is a GDP-activated tetrasaccharide (Table 1). Both oligosaccharides contain additional GlcNAc residues compared with the intact glycan of the mature S-layer glycoprotein (3).

Isolation and characterization of a lipid-activated intermediate. The butanol extracts (9) of cells disrupted by shaking with glass beads were applied to silica plates (solvent e). Bands were visualized with iodine vapor, eluted, and subsequently run on silica plates for a second time, using solvent g. One lipid- and ManNAc-containing band could be purified (Table 1, compound 7). Quantitative and structural analyses of compound 7 (Table 1 and 2) were performed as described for compounds 1 and 2. The results indicate that compound 7 is a dolichyl-activated hexasaccharide consisting of the repeating trisaccharide unit of the intact glycan (3) and three additional GlcNAc residues. As only one GlcNAc residue was destroyed with periodate, the three GlcNAc residues should be linearly arranged in a 1→3 or 1→4 linkage. The lipid carrier of compound 7 was identified as dolichol-C_{55} by cochromatography with standards as described recently (8) and by mass spectrometry after splitting off of the carbohydrate and phosphate residues (hydrolytic condition g) (8).

As shown in Fig. 2, the mass spectrometric analysis revealed a protonated molecular ion ($[M+H]^+$) at *m/z* 770.26, a sodiated molecular ion ($[M+Na]^+$) at *m/z* 792.58, and a cluster molecular ion ($[M+PAA+H]^+$) with high abundance at *m/z* 919.75, consistent with the dolichol-C_{55} structure. The calculated average relative molecular mass of dolichol-C_{55} is 769.35. The relative molecular mass was determined to be 769.40 (+0.0065%), calculated as the average of those for $[M+H]^+$, $[M+Na]^+$, and $[M+PAA+H]^+$. Additionally, the negative-ion plasma desorption mass spectrum (not shown) exhibited a deprotonated molecular ion ($[M-H]^-$) at *m/z* 767.79 and a deprotonated cluster molecular ion ($[M+PAA-H]^-$ at *m/z* 917.94. Weak signals at *m/z* 850.14, 872.48, and 999.75 were also present. These ions correspond to the protonated and sodiated molecular

TABLE 1. Analysis of isolated nucleotide-activated precursors (compounds 1 to 6) and an isolated lipid-activated oligosaccharide (compound 7)

Compound no.	Sephadex column peak no.[a]	TLC (R_f)					Absorption		Dolichol-C_{55}	Composition (molar ratio)							Proposed structure[b]
		Solvent a	Solvent b	Solvent c	Solvent e	Solvent f	260/280 nm	250/260 nm		Guanine	Uracil	Phosphate	ManNAc	GlcNAc	Glc	Gal	
1	I	0.35[b]	0.82	0.24[b]	ND	ND	0.69	0.99	—	1.0	—[c]	2.3	1.1	1.0	—	0.9	GDP–ManNAc←Gal←GlcNAc ↕ ManNAc ↕ Gal ↕ GlcNAc
2	I	0.10[b]	0.75	0.09[b]	ND	ND	0.71	1.03	—	1.0	—	2.0	0.9	0.9	1.0	0.9	GDP–ManNAc←Gal←GlcNAc ↕ Glc ↕ ManNAc←Glc ↕ Gal ↕ GlcNAc
3	II	0.50	ND[d]	0.25	ND	ND	0.42	0.78	—	—	1.0	2.1	—	—	1.1	—	UDP–Glc ↕ Glc
4	II	0.49	ND	0.22	ND	ND	0.40	0.79	—	—	1.0	2.0	—	—	—	0.9	UDPGal ↕ Gal
5	II	0.50	ND	0.32	ND	ND	0.61	0.96	—	1.0	—	1.9	1.2	—	—	—	GDP–ManNAc ↕ ManNAc
6	II	0.51	ND	0.33	ND	ND	0.40	0.80	—	—	1.0	2.1	—	0.8	—	—	UDP–GlcNAc ↕ GlcNAc
7		ND	ND	ND	0.42	0.49	ND	ND	Present	—	—	2.04	1.18	3.20	1.00	0.91	Dolichol-$_{C55}$–PP_i–ManNAc←Gal←GlcNAc←GlcNAc←GlcNAc ↕ Glc

[a] See Fig. 1.
[b] See Table 2.
[c] —, not present.
[d] ND, not determined.

TABLE 2. Analysis of purified oligosaccharides obtained after partial hydrolysis of the isolated precursors

Compound no.[a]	Derived oligosaccharide no.	R_f[b]	Composition (molar ratio)				Proposed structure
			ManN	GlcN	Gal	Glc	
1	1a	0.44	1.0[c]	—[d]	0.9	—	ManNAc←Gal
	1b	0.34	—	1.0	1.0[c]	—	Gal←GlcNAc
2	2a	0.45	1.0[c]	—	1.1	—	ManNAc←Gal
	2b	0.40	1.0[c]	—	—	1.2	ManNAc←Glc
	2c	0.35	—	0.9	1.0[c]	—	Gal←GlcNAc
7	7a	0.45	1.0[c]	—	1.1	—	ManNAc←Gal
	7b	0.41	1.0[c]	—	—	1.2	ManNAc←Glc
	7c	0.35	—	0.9	1.0[c]	—	Gal←GlcNAc
	7d	0.45	—	1:1[e]	—	—	GlcNAc←GlcNAc
	7e	0.27	—	1:2[e]	—	—	GlcNAc←GlcNAc←GlcNAc

[a] See Table 1.
[b] Value in solvent d after partial hydrolysis and N acetylation of the amino sugars.
[c] Reducing sugar (determined as described in Materials and Methods).
[d] —, not present.
[e] Molar ratio of reducing sugar (=1) to nonreducing sugar.

ion as well as to the cluster molecular ion ($[M+PAA+H]^+$) of the dolichyl-C_{55} monophosphate. This observation clearly indicates incomplete hydrolysis of the sample and also helps to verify the precursor structure. However, no fragment ions at this level of deposited sample amount (approximately 5 μg) could be detected.

DISCUSSION

From analysis of the isolated precursors, we believe that the biosynthesis (Fig. 3) of the S-layer glycoprotein of *B. alvei* starts with the formation of nucleotide-activated monosaccharides of Gal, Glc, ManNAc, and GlcNAc. The nucleotide is GDP in the case of ManNAc and UDP in the case of the other sugars. Three of the nucleotide-activated monosaccharides (GDP-ManNAc, UDP-Gal, and UDP-GlcNAc) are linked together, forming a GDP-activated trisaccharide with ManNAc at the reducing end. In the next step, UDP-Glc is transferred to the trisaccharide, forming a GDP-activated branched tetrasaccharide. This tetrasaccharide shows the same structure as does the repeating unit of the intact glycan (3), but it contains one additional GlcNAc residue. In a next step, the GDP residue of the tetrasaccharide should be replaced by dolichyl-C_{55}–PP_i. This lipid-activated precursor contains two additional GlcNAc residues compared with the GDP-activated tetrasaccharide. Whether the GlcNAc residues are added before or after the transfer of the oligosaccharide to the lipid remains to be determined. No GlcNAc is found in the intact glycoprotein (3), which indicates that the additional GlcNAc residues are removed during further processing.

Transient occurrence of sugar residues is also the case with eukaryotic glycoproteins (4), the S-layer glycoprotein of *Methanothermus fervidus* (8), and the S-layer glycoprotein of *Halobacterium halobium* (15). Like undecaprenol, the dolichol derivative consists of 55 carbons. It is smaller than most eukaryotic dolichol derivatives but of the same size as the one found in *M. fervidus* (8). The proposed pathway has to be verified by further studies with labeled compounds and enzyme preparations.

Recently, nucleotide-activated oligosaccharides were proposed to be involved in the biosynthesis of different cell envelope polymers (glycoprotein [8], pseudomurein [9], and polysaccharide [10]) of methanogens. Therefore, the formation of nucleotide-activated oligosaccharides seems to be a typical and common feature of the biosynthesis of methanobacterial cell wall glycans, while in eubacteria, they occur only as intermediates in glycoprotein biosynthesis.

Since dolichol functions as a lipid carrier in the biosynthesis of two archaebacterial glycoproteins (8, 14), a eubacterial glycoprotein, and eukaryotic glycoproteins (4), it seems most likely that dolichol is the universal lipid carrier for glycoproteins, while undecaprenol is the common lipid carrier of other cell wall glycans (9, 10, 20).

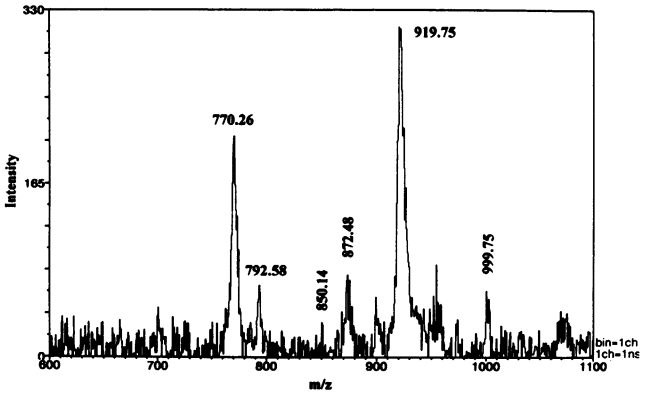

FIG. 2. Positive-ion plasma desorption mass spectrum of the dolichyl monophosphate fraction after partial hydrolysis.

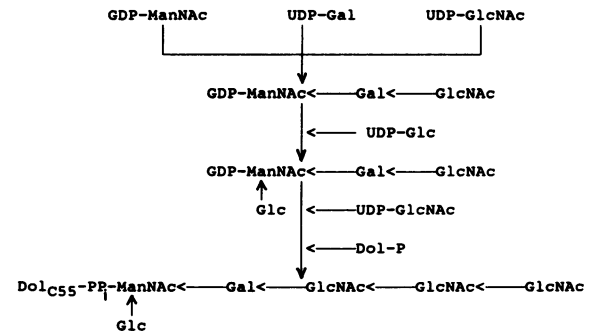

FIG. 3. Proposed scheme of the biosynthesis of the S-layer glycoprotein of *B. alvei*. Dol, dolichyl; Dol_{C55}, dolichyl-C_{55}.

Except for a saturated α-C-C bond, dolichol-C_{55} has the same structure as does undecaprenol. Nevertheless, the consequences for the further biosynthetic route of nucleotide-activated precursors are significant.

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