Isolation and characterization of dolichol-linked oligosaccharides from *Haloferax volcanii*

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**Introduction**

The surface layer glycoprotein from the extreme halophile *Halobacterium halobium* was the first prokaryotic glycoprotein to be discovered (Mescher and Strominger, 1976). Structural work from our laboratory elucidated the complete chemical structure of this glycoprotein (for a review see Sumper, 1987; Lechner and Wieland, 1989; Sumper and Wieland, 1995). Two different types of sulfated saccharides are linked to the polypeptide in N-glycosyl bond. The biosynthetic precursors of these saccharides have been characterized. Dolichol monophosphate-linked intermediates are involved in the biosynthesis of the sulfated oligosaccharides, and a lipid-diphosphate (most probably a dolichol derivative) serves as the saccharide carrier for the repeating unit saccharide. The flagellar bundles of *Halobacterium halobium* consist of five different flagellins that turn out to be glycoproteins as well (Wieland et al., 1986). The structure of the related surface layer glycoprotein from the moderate halophile *Haloferax volcanii* has been studied in some detail to get hints on how adaptation to high salt condition is achieved. Interestingly, the acidic saccharides found in the extreme halophile are replaced by neutral saccharides in the moderate halophile (Mengele and Sumper, 1992).

There is some evidence that additional glycoproteins with unknown functions exist in *H. volcanii* (Zhu et al., 1995). Up to now, no biosynthetic precursors for the glycosylation pathways in *H. volcanii* have been described. In this article, we describe the purification and characterization of three dolichol-linked oligosaccharides.

**Results**

**Isolation of glycolipids**

To isolate dolichol-linked oligosaccharides, cells of *Haloferax volcanii* were extracted according to the procedure of Folch et al. (1957). Dolichol activated saccharides should be found in the chloroform-methanol extract along with phospholipids and glycolipids (Torreblanca et al., 1986). As membranes from archaea contain glycerol ether lipids which resist hydrolysis under mild alkaline conditions, it is difficult to separate glycolipids from dolichol-linked saccharides. To remove less polar lipids (e.g., carotinoïdes), the extract was first fractionated by silica gel column chromatography. The fraction containing polar lipids was further separated by high performance liquid chromatography on a silica gel column. The dominant glycolipid, sulfated diglycosyl diether (Torreblanca et al., 1986), elutes well separated from the dolichol-linked oligosaccharides. Aliquots of each fraction were analyzed by thin layer chromatography (Figure 1). Three orcinol positive compounds were pooled as indicated (fraction I, fraction II, and fraction III) in Figure 1. From 70 g wet cells, the yield was about 200 μg of compound I, about 4 μg of compound II and about 50 μg of compound III. In some preparations, phosphodolichol eluted in tube 36 contributed to only a minor contamination of fraction I, insufficient to affect subsequent analysis. Fraction II appears to contain two compounds, but further structural analysis (see below) only indicated the presence of a single glycolipid species. Therefore, it is not clear why compound II provides two spots on TLC. The minor contaminants of fraction III appeared not to interfere with subsequent structural analysis (see below).

**Hydrolysis of lipid-linked oligosaccharides**

To examine whether these compounds indeed represent dolichol-linked saccharides, they were submitted to mild acid hydrolysis (Lechner et al., 1985). After partitioning the resulting products between chloroform and water, both the water phase containing any released saccharides and the chloroform phase containing dolichol phosphate were analyzed by thin layer chromatography. The water phases contained oligosaccharides which exhibited lower Rf values than the untreated compounds (Table I). In each case, phosphodolichol was detected in the chloroform phase with a Rf value of 0.54. In addition, the presence of phosphate in the chloroform phases was demonstrated by the colorimetric assay described by Ames (1966).
Characterization of the oligosaccharide moieties
The released oligosaccharides were further purified by anion exchange chromatography. As shown in Figure 2, only one major oligosaccharide was present in each of the three fractions (designated as peak I to III). The sugar composition was determined by GC/MS (Figure 3): peak I saccharide contained equal amounts of galactose and mannose, whereas peak III saccharide contained mannose, galactose and rhamnose in the ratio of 2:1:1. Analysis of peak II saccharide was not possible due to the small amount of material available.

The oligosaccharide released from compound I was further analyzed by permethylation analysis according to Hakomori (1964) (Figure 4). After hydrolysis, two partially methylated hexitol derivatives were obtained. By comparison of the retention times and the MS-spectra with those derived from lactose after permethylation and hydrolysis, the material in peak A was identified as a C-4-linked galactosyl residue. By comparison with reference spectra (Jansson et al., 1976), the peak B material was identified as being derived from a terminally bound hexose residue. Thus, the corresponding oligosaccharide is the disaccharide mannose (1→4) galactose.

The oligosaccharides derived from the material in peak II and III were subjected to mass spectrometry (Figure 5). In the spectrum of peak III saccharide, a signal with the \( m/z \) value of 729.2 was detected which is consistent with the molecular weight of a phosphorylated or sulfated tetrasaccharide containing three hexoses and one deoxyhexose (theoretical \( M_r \), 729.6). This tetrasaccharide was not derivatized at the anomeric C-atom as the molecular weight of peak III saccharide increased to 732.5 after reduction by NaBD\(_4\) (data not shown). Peak II saccharide exhibited a signal of \( m/z \) 421.0 indicating the presence of a sulfated or phosphorylated disaccharide of two hexoses (theoretical \( M_r \), 421.3). To little material was available for a further analysis of this latter saccharide.

Characterization of the lipid moieties
The lipid moieties recovered in the chloroform phases after mild acid hydrolysis of fraction I to III were also analyzed by mass spectrometry (Figure 6). The lipid moieties from all three compounds produced two signals with \( m/z \) of 850.0 and 918.2. These values are consistent with the calculated molecular weight of a phosphodolichol with a chain length of 11 and 12 isoprene units (theoretical \( M_r \), 847.3 and 915.4), respectively. The difference of 2 \( m/z \) in each case indicates the presence of a second dihydro isoprene unit.
Dolichol-linked oligosaccharides from *Halofera volcanii*

![Graphs showing GC-mass spectrometric analysis of saccharides of peak I and peak III as alditol acetates](image)

**Fig. 3.** GC-mass spectrometric analysis of the saccharides of peak I and peak III as alditol acetates; (total ion profile). The carbohydrate moieties of peak I saccharide (A) and peak III saccharide (B) were hydrolyzed, reduced, and peracetylated. Details are given under Materials and methods. (A) Mass peak profile of peak I saccharide on Hewlett Packard GC/MS; (B) mass peak profile of peak III saccharide on Finnigan MAT Magnum GC/MS.

**1H-NMR spectra of mannosylgalactosyl phosphodolichol**

Only the glycolipid of fraction I produced sufficient amounts of material to take 1H-NMR spectra in order to determine the anomeric configuration of the glycosidic linkage and to confirm the dolichol structure of the lipid moiety. The data obtained are summarized in Table II.

From the chemical shift values and from the coupling constants for the H-1 protons of galactose and mannose the anomeric configuration of both sugars could be analyzed unequivocally. Mannose is β-glycosidically bound to galactose and the latter residue is bound in α-configuration to the lipid phosphate. The values of the adjacent sugar protons confirm this configuration (Hounsell, 1995). The number of isoprene

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![Graphs showing purification of carbohydrate moieties by anion exchange chromatography.](image)

**Fig. 2.** Purification of the carbohydrate moieties by anion exchange chromatography. The saccharide compounds of fraction I to fraction III released by mild acid hydrolysis were purified by anion-exchange high performance liquid chromatography under strong alkaline conditions. The eluent was monitored by pulsed amperometry. An aliquot of fraction I and fraction III was applied whereas for fraction II nearly the total amount of the sample was analyzed. For details, see Materials and methods. (A) fraction I (Figure 1); (B) fraction II; (C) fraction III.
units forming the polyisoprene moiety could be derived by integration and calculation of significant isoprene hydrogens. The presence of saturated isoprene units is confirmed by the resonances and signal intensities at 0.87, 0.92, and 1.15 to 1.49 ppm (Jaenicke and Siegmund, 1989). The presence of two saturated isoprene units was also indicated by the corresponding mass spectra.

**Mass spectra of the intact dolichol-linked oligosaccharides isolated**

Finally, mass spectra of the intact compounds I, II, and III were taken to confirm the postulated structures (Figure 7). Compound I exhibits ion peaks of m/z 1175.5 and m/z 1242.5 consistent with the theoretical molecular weight of a dihexosyl phosphodolichol of 11 and 12 isoprene units with α- and ω-saturation (theoretical M, 1175.3 and 1243.5). These results identify compound I as mannose (β1→4) galactosyl (α) phosphodolichol.

Compound II exhibits two signals of m/z 626.8 and 660.6. By taking together the data for the molecular weights of the oligosaccharide moiety (M, 421.0, Figure 5B) and the lipid moiety (M, 850.3 and 919.2) a molecular weight of 1253.3 and 1322.2 is predicted. The observed molecular weight values (626.8 and 660.6) must be doubled as there are two charged groups in the intact compound II, confirming the structure of compound II as a sulfated or phosphorylated dihexosyl saccharide linked to a phophodolichol of 11 and 12 isoprene residues.

Compound III reveals two ion peaks of m/z 781.0 and m/z 814.8. The charged tetrasaccharide moiety was shown to have a molecular weight of 729.2 (Figure 5A), the lipid moiety a molecular weight of 850.0 and 918.2 (Figure 6) which adds to molecular weights of 1562.1 and 1630.3 for the parent molecule. Again, the presence of two charged groups require the doubling of the measured values as z = 2. The observed molecular weights of 1562.0 and 1629.6, respectively (theoretical M, 1561.9 and 1630.3), confirm that compound III consists of a sulfated or phosphorylated tetrasaccharide being composed of three hexoses and one deoxyhexose which is linked via a phosphate bridge to a dolichol with 11 or 12 isoprene units.

**DEAE-chromatography**

The presence of an additional charged group in compound III as compared to compound I was further demonstrated by chromatography on a DEAE-cellulose column. After elution of the column with increasing concentrations of ammonium formate the desalted and concentrated eluates were analyzed by tlc. Compound I was eluted with 25 mM ammonium formate,
whereas compound III required 100 mM ammonium formate for elution.

**Characterization of dolicholphosphate**

The noncarbohydrate compound present in our preparation (Figure 1) was analyzed by thin layer chromatography, $^1$H-NMR spectroscopy, and mass spectrometry and identified as free dolicholphosphate with 11 and 12 isoprene residues with saturated $\alpha$- and $\omega$-units.

**Discussion**

The biosynthesis of procaryotic glycoproteins has been investigated in detail in *Halobacterium halobium*. Lechner et al. identified the lipid intermediates and concluded that these archaea use dolichol like eucaryotes rather than undecaprenol (Lechner et al., 1985). Furthermore, phosphodolichol linked oligosaccharides as well as diphosphodolichol linked oligosaccharides were shown to be involved in protein glycosylation. Now we confirm the occurrence of phosphodolichol linked oligosaccharides in the moderate halophile *Haloferax volcanii*. In the present study, relatively hydrophobic conditions were used for extraction, and this might explain that we did not detect oligosaccharides linked via a diphosphate bridge to dolichol. Halobacteria share with eucaryotes the use of dolichol as a saccharide carrier. But there are some differences. First, as revealed by MS-analysis and $^1$H-NMR spectroscopy, in the dolichol from *H. volcanii* the ultimate isoprene unit is also saturated. There are only two reports of eucaryotic dolichols with additional dihydro isoprene residues (Stone et al., 1967; Barr and Hemmings, 1972). Interestingly, in these cases it is also the $\omega$-terminal isoprene unit that is found to be saturated. Secondly, in *H. volcanii*, the oligosaccharides are found to be linked via a monophosphate bridge, whereas in eucaryotes oligosaccharides are exclusively linked to diphosphodolichol. Finally, the halobacterial cells possess a limited spectrum of dolichol species containing only 11 or 12 isoprene units. This is in contrast to the broad spectrum of long chain dolichols (14–23 isoprene residues) synthesized by eucaryotic cells (Chejnacki and Dallner, 1988). The presence of unusual short chain dolichols was previously reported in the protozoon *Trypanosoma brucei* (Löw et al., 1991).

As the saccharide-1-phosphate linkage has a transfer potential comparable to those of nucleotide-diphosphate sugars (Hemmings, 1985), it is very likely that the dolichol linked oligosaccharides described here will transfer their saccharide moiety to a suitable protein acceptor. Up to now, only the S-layer glycoprotein has been characterized and shown to contain two types of saccharides: O-linked disaccharides (glucosyl (a12) galactose) and N-linked oligosaccharides containing mainly $\beta$-1,4-linked glucose residues as well as a few galactose and idose residues (Sumper et al., 1990; Menegle and Sumper, 1992). Total sugar analysis of the S-layer glycoprotein, however, reveals that mannose, galactose, glucose and idose are

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**Table II. Signal assignment in 1D and 2D $^1$H-NMR spectra of fraction I**

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Shift (ppm)</th>
<th>Coupling constant (Hz)</th>
<th>Number of protons</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_3$-saturated-$\omega$</td>
<td>0.87</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>CH$_3$-saturated-$\alpha$</td>
<td>0.92</td>
<td>6.5</td>
<td>3</td>
</tr>
<tr>
<td>CH$_2$-CH$_2$-CHR-CH$_3$</td>
<td>1.15–1.49</td>
<td>s</td>
<td>10</td>
</tr>
<tr>
<td>CH$_3$-trans</td>
<td>1.56–1.61</td>
<td>s</td>
<td>9</td>
</tr>
<tr>
<td>CH$_2$-cis</td>
<td>1.68</td>
<td>s</td>
<td>21</td>
</tr>
<tr>
<td>CH$_3$-C = C</td>
<td>2.0–2.1</td>
<td>m</td>
<td>40</td>
</tr>
<tr>
<td>CH$_3$-O-CH$_3$</td>
<td>3.96</td>
<td>m</td>
<td>2</td>
</tr>
<tr>
<td>C = CH</td>
<td>5.15</td>
<td>m</td>
<td>10</td>
</tr>
</tbody>
</table>

**Chemical shift (coupling constant)-ppm (Hz)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>H-1</th>
<th>H-2</th>
<th>H-3</th>
<th>H-4</th>
<th>H-5</th>
<th>H-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>(B-1-4)Gal(α1-P)</td>
<td>5.52</td>
<td>3.84</td>
<td>3.91</td>
<td>4.18</td>
<td>4.06</td>
<td>3.63, 3.85</td>
</tr>
<tr>
<td></td>
<td>dd</td>
<td>dd</td>
<td>dd</td>
<td>dd</td>
<td>dd</td>
<td>dd</td>
</tr>
<tr>
<td>J$_{1,2}$</td>
<td>3.5</td>
<td>10</td>
<td>3</td>
<td>0.5</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>J$_{1,2}$</td>
<td>7</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Man(B1-)</td>
<td>4.80</td>
<td>4.04</td>
<td>3.43</td>
<td>3.49</td>
<td>3.22</td>
<td>3.67, 3.91</td>
</tr>
<tr>
<td></td>
<td>dd</td>
<td>dd</td>
<td>dd</td>
<td>dd</td>
<td>dd</td>
<td>dd</td>
</tr>
<tr>
<td>J$_{1,2}$</td>
<td>3.5</td>
<td>9</td>
<td>5</td>
<td>3</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>J$_{1,2}$</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>9</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

Chemical shifts are given downfield from tetramethyl silane as internal standard. The solvent used was CD$_3$OD.

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**Fig. 6. Mass spectrometry of the lipid moieties of fraction I, II, and III. Spectra of the lipid moieties of fraction I, II, and III (obtained after mild acid hydrolysis and partitioning into the chloroform phase) were taken on Finnigan MAT 900 by electrospray ionization with negative ion detection. All samples produced identical signals; therefore, only the mass spectrum derived from the material of fraction I is shown.**

**Dolichol-linked oligosaccharides from Haloferax volcanii**
Halobacterium mediterranei recently detected in C.Kuntz et al. (1990). Extracellular polysaccharides may be additional candidates as saccharide acceptors. An extracellular polysaccharide was re-isolated from the marmosylgalactose disaccharide could serve as a precursor. Therefore, additional glycosylation sites may exist for which unknown function may exist in H. volcanii (Kuntz et al., 1995). Cells were harvested in the late log-phase using a continuous flow centrifuge and stored at -20°C.

**Materials and methods**

**Organism and cultivation**

_Haloferax volcanii_ (DSM 3757) was grown in 50 l batches in complex medium as described by Muhlakhanbahi and Larsen (1975). Cells were harvested in the late log-phase using a continuous flow centrifuge and stored at -20°C.

**Extraction of lipids**

Lipids were extracted according to the method of Folch et al. (1957). After thawing, 50 g of cells (wet weight) were suspended in 200 ml of cold methanol and stirred for 30 min at 4°C. Then 300 ml of CHCl₃ was added and lipids were extracted by stirring for another 45 min. Finally the suspension was centrifuged (16,300 x g for 30 min at 4°C). The resulting supernatant was washed with 100 ml of water. Phases were separated by centrifugation. The lower phase was washed three times with the theoretical upper phase described by Folch et al. (1957) and then dried by evaporation. This extract is called chloroform-methanol extract.

**Purification of lipid-linked saccharides**

The dried chloroform-methanol extract was dissolved and submitted to chromatography on a silica gel column (silica gel 60, 0.063-0.2 mm, Merck, Darmstadt, 3.9 x 5 cm) according to Wells et al. (1981) in solvent A (CHCl₃/CH₃OH/25%NH₄OH, 65:29:6). After washing with four bed volumes of solvent A, the column was eluted with solvent B (CHCl₃/CH₃OH/25% NH₄OH, 52.5:35.5:6:6).

The dried effluent was redissolved in solvent A and submitted to high performance liquid chromatography on silica gel (25 cm x 1 cm, Lichrosorb, Si 60, 7 µm, Merck, Darmstadt) (Wells et al., 1981). After washing with 240 ml of solvent A a linear gradient from 0% to 100% solvent B was applied for 60 min at a flow rate of 4 ml/min. Aliquots of each fraction were run on thin layer chromatography plates and tested for saccharides by spraying with the orcinol reagent (Vaskovsky et al., 1979).

**Thin-layer chromatography**

Thin-layer chromatography was carried out on silica gel 60 F₂₅₄ aluminum sheets (Merck, Darmstadt) using one of the following solvent systems: A, butanol/ethanol/water (5:3:2, by volume); B, propan-1-ol/water (65:35, by volume); C, chloroform/methanol/acetic acid/water (70:18:5:7:0.5, by volume). Following chromatography lipids were detected by iodine vapor, saccharides were visualized by the orcinol reagent.

**Acid hydrolysis**

For mild acid hydrolysis, dolichol-linked sugars were dissolved in 40 µl water/propan-1-ol (1:1, by volume) and incubated in 400 µl 10 mM HCl for 10 min at 95°C. 60 µl CHCl₃ was added, and the phases were separated by centrifugation. The lower phase was washed two times with 500 µl water. The combined water phases contained the released saccharides.

**Quantification of phosphate**

Phosphate was determined with the colorimetric assay described by Ames (1966).

**Purification of the saccharides**

Saccharides were further purified by high performance anion-exchange chromatography with pulse-amperometric detection. The analyses were carried out on a Dionex-BioLC-system connected with a Carbo Pac PA1 column (4 mm x 250 mm) and a guard column PA (3 mm x 25 mm). The flow rate was...
1 ml/min. For detection the following pulse potentials and durations were used: 
\[ \begin{align*} 
E_1 &= 0.10 V (500 \text{ ms}), \\
E_2 &= 0.60 V (100 \text{ ms}), \\
E_3 &= -0.60 V (50 \text{ ms}). 
\end{align*} \]
Eluent A was 150 mM NaOH; eluent B was 150 mM NaOH with 500 mM sodium acetate. Chromatography conditions were 2% B for 5 min, and then 2% B to 100% B in 2 min. The eluted saccharides were immediately neutralized by concentrated acetic acid and desalted by passage through a small column containing 1 ml of anion exchange resin (AG-X4, free form, Bio-Rad) and 1 ml of cation exchange resin (AG50 W-X8 H+) in water. The eluent was lyophilized.

Reduction of oligosaccharides

The dried oligosaccharide was incubated in 0.1 M NaOH/1M NaBD\(_4\) for 30 min at 37°C. After neutralization with 5 M acetic acid, the sample was applied to a column of Dowex AG50 W-X8 H+ (1 ml) (Bio-Rad). The eluate was dried. Borate was removed by the addition of methanol and subsequent evaporation. This step was repeated twice.

Alditol acetates

After hydrolysis with 4 M trifluoracetic acid for 4 h at 100°C, the monosaccharides were converted to alditol acetates according to Spro (1972).

Permethylation

Permethylation of the saccharides was performed according to the method of Hakomori (1964), as modified by Waeghe et al. (1983). The sodium dimethylsulfynil carbamion was prepared as described by Harris et al. (1984). The permethylated saccharide was purified with Sep-Pak C\(_18\) cartridges (Waters Millipore Inc.) by elution with 25% acetonitrile. After hydrolysis in 4 M trifluoracetic acid for 4 h at 100°C, the methylated sugars were reduced with NaBH\(_4\), peracetylated, and analyzed by GC/MS.

DEAE-Chromatography of dolichol-linked oligosaccharides

The dolichol-linked oligosaccharides were dissolved in CHCl\(_3\)/methanol (3:2) and applied to a column (1.4 x 1 cm) of DEAE-Sephalac (acetate form) (Pharmacia), equilibrated with the same solvent. After washing with 6 bed volumes of CHCl\(_3\)/methanol (3:2) the column was eluted by stepwise increasing concentrations of ammonium formate in the same solvent. To remove residual ammonium formate from the dried effluent, the material was dissolved in 5 ml CHCl\(_3\)/methanol (3:2) and washed with 1 ml of water. After phase separation, the lower phase was washed with 1 ml theoretical upper phase according to Polch et al. (1957) and analyzed by tic.

Acknowledgments

We thank R. Deutzmann and E. Hochmuth for taking the mass spectra and H.-D. Lüdemann for valuable advice and discussions.

Abbreviations

GC/MS, gas chromatography; mass spectrometry; HPLC, high performance liquid chromatography.

References


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