Primary Structure of the Heterosaccharide of the Surface Glycoprotein of *Methanothermus fervidus*

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Uwe Kärcher†, Harald Schröder‡, Ernst Haslinger§, Günter Allmaier‖, Felix Wieland¶, Anton Haselbeck** and Helmut König††

From the †Abteilung Angewandte Mikrobiologie und Mykologie der Universität Ulm, 0-89069 Ulm, Germany, the ‡Abteilung Organische Chemie I der Universität Ulm, D-89069 Ulm, Germany, the ††Institut für Biochemie I, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany, **Boehringer Mannheim GmbH, Werk Penzberg, D-82372 Penzberg, Germany, and the ‡‡Institut für Analytische Chemie der Universität Wien, Währinger Str. 38, A-1090 Wien, Österreich

The outer surface of the cells of the hyperthermophile *Methanothermus fervidus* is covered by crystalline glycoprotein subunits (S-layer). From the purified S-layer glycoprotein, a heterosaccharose was isolated. The heterosaccharose consists of D-3-O-methylmannose, D-mannose, and D-N-acetylgalactosamine in a molar ratio of 2:3:1 corresponding to a molecular mass of 1061.83 Da. 3-O-methylmannose could be partly replaced by 3-O-methylglucose. The primary structure of the glycan was revealed by methylation analysis, by plasma desorption mass spectrometry, and by high field NMR spectroscopy. The purified heterosaccharose is linked via N-acetylgalactosamine to an asparagine residue of the peptide moiety. The following structure is proposed for the heterosaccharose: α-D-3-O-MetManp-(1→6)-α-D-3-O-MetManp-(1→2)-α-D-Manp3-(1→4)-β-D-GalNAc.

Since the first report on the occurrence of glycoproteins in procaryotes, the chemical structure of several archaeal and bacterial surface layer glycoproteins has been determined in recent years (1–3). The S-layer of the hyperthermophilic methanogen *Methanothermus fervidus* also consists of glycoprotein subunits (4), which are situated outside of the pseudomurein sacculus. The peptide of the mature glycoprotein is composed of 593 amino acid residues corresponding to a molecular mass of 65 kDa (5). The carbohydrate moiety mainly consists of 3-O-methylmannose, mannose, and N-acetylgalactosamine. 3-O-methylglucose, N-acetylgalactosamine, and galactose were also found in lesser amounts in intact glycoprotein preparations. Compared with other mesophilic S-layer glycoproteins, this hyperthermophilic surface glycoprotein possesses significantly higher amounts of isoleucine, asparagine, and cysteine (5). It also has 14% more β-sheet structures. Asparagine occurs often in Asn-Asn-Xaa-Ile clusters, where isoleucine could be partly replaced by tryptophane, phenylalanine, or glycine (5). The amino acid sequence derived from the encoding gene revealed the presence of a typical leader peptide and 20 sequon structures (Asn-Xaa-Thr/Thr) as potential carbohydrate binding sites. The previous investigations on the biosynthesis of the glycan moiety revealed the involvement of nucleotide-activated oligosaccharides and C5-dolichol as lipid carrier (6).

In order to characterize this hyperthermophilic surface glycoprotein of *M. fervidus* in more detail, we report here on the isolation and structure of a heterosaccharide.

MATERIALS AND METHODS

Bacterial Strain

A stock culture of *M. fervidus* strain V24S (7) (DSM 2068) was provided by R. O. Stetter (Lehrstuhl für Mikrobiologie, Universität Regensburg). The cells were grown in a 10-liter glass fermenter at 84 °C in medium 1 (as described in Ref. 8). The cell culture medium was constantly supplied with H2S (0.5 ml/min). The cells were harvested with a centrifuge (Padberg) at an optical density of about 2.5 (578 nm). The cell pellet was stored at −70 °C.

Isolation of the S-layer Glycoprotein

The S-layer glycoprotein was extracted with trichloracetic acid as described (4) and subsequently dialyzed.

Carbohydrate Analysis

Neutral and amino sugars were identified as alditol acetates (9) by gas-liquid chromatography (Hewlett Packard, model 5880A) on a Durabond 1701 capillary column (25 m, I.C.T. Laboratories). Quantitative analysis of amino sugars was carried out with an amino acid analyzer (Biotronic LC 5000).

Hydrazinolysis, Acetylation, and Reduction

The glycan chains were split off from the peptide by hydrazinolysis (11, 12). The method was modified according to instructions of Oxford GlycoSystems (Oxford; 3.75 h, 95 °C). N-acetylation and reduction of the carbohydrate were done as described by Montreuil et al. (12).

Separation of O-linked Carbohydrates

The occurrence of O-linked glycan chains in the glycoprotein was tested by a selective method for splitting off O- and N-linked glycans with NaBH4 and cadmium acetate (13).

Column Chromatography

Fractogel TSK HW40 S Column (22 mm × 600 mm, Merck)–H2O was used as eluent. The flow rate was 1 ml/min. The elution profile was recorded at 220 nm. Fractions of 3 ml were collected.

Bio-Gel P-2 Column (16 mm × 700 mm, Bio-Rad)—The eluant was H2O with a flow rate of 0.7 ml/min. The elution profile was recorded by a refractive index monitor (Bio-Rad Model 1755). Fractions of 1.4 ml were collected.

High Performance Liquid Chromatography—For high performance liquid chromatography, the system 400 of Kontron was used. The following columns were applied for purification of the heterosaccharide: (a) ProRPC HR 18/10 silica gel C2-C8 reversed phase column...
Thin Layer Chromatography
Silica gel 60 (thin layer chromatography aluminium sheets, Merck) was used. Solvent system was as follows: butanol:ethanol:H₂O (1:1:1). The silica gel plates were incubated at 100 °C for 15 min.

CrO₃ Oxidation
5 mg of the purified glycoprotein were treated with CrO₃ according to Hoffmann et al. (14).

Methylation Analysis
The methylation analysis (15) was performed using a combined gas-liquid chromatography and mass spectrometry system (Hewlett Packard 5995). Characteristic masses and the relative intensity of the signals were compared with reference gas-liquid chromatography/mass spectrometry spectra (16).

Periodate Oxidation
The periodate oxidation was performed with the isolated glycoprotein (17).

NMR Spectroscopy
NMR spectra were recorded with a Bruker AMX-500 spectrometer (¹H frequency, 500.13 MHz; ¹³C frequency, 125.76 MHz). Data processing was achieved with an Aspect X32 computer by using UXNMR software. 5-mm reverse probe head was as follows: solvent, D₂O/methanol-d₄ 1:1; temperature, 300 K. The methanol signal was used as an internal standard (³H, δ = 3.3; ¹³C, δ = 49.0). The parameters were as follows: 90° pulses (H, 9.6–10 μs; ¹³C, 10.3 μs; WALTZ, 'H-decoupling pulse, 112 μs; GARP, ¹³C-decoupling pulse, 80 μs; MLEV-17 pulse, 20.5 μs; COSY, 45° mixing pulse; TOCSY, phase-sensitive using TPP1, mixing time 120 ms (100 MLEV-17 cycles plus two trim pulses of 2.5 ms each); ROESY, phase-sensitive using TPP1, spinlock cw pulse (250 ms); HMQC, phase-sensitive using TPPI, BIRD sequence, GARP-decoupled; HMQC-TOCSY, phase-sensitive using TPPI, mixing time 120 ms (100 MLEV-17 cycles plus two trim pulses of 2.5 ms each)). The delay for an optimal magnetization transfer was adjusted to 1/3.

Determination of the Configuration
The configuration ofmannose and the 3-O-methylhexoses was determined enzymatically (10). The 3-O-methylhexoses were demethylated before enzyme treatment. The 3-O-methylated hexoses of the glycan were de-O-methylated according to Hough and Theobald (18). The de-O-methylated monosaccharides were identified by gas-liquid chromatography analysis as their alditol acetates. In the case of galactosamine, the configuration was derived from its degradation product series (19).

Acetylation
Acetylation was performed according to Lindberg et al. (20). The products were separated on an Aminex HPX 42A column. The eluent was H₂O. The flow rate was 0.4 ml. The carbohydrate-containing fractions were hydrolyzed and analyzed as alditol acetates.

Mass Spectrometry
Mass spectra were obtained on a short linear time-of-flight mass spectrometer equipped with a Californium-252 plasma desorption source (Applied Biosystems, Foster City, CA). The length of the field-free drift region was 141 mm and the typical pressure 1.0 × 10⁻⁵ Pascal. The applied acceleration voltage was 19 kV in the positive ion mode. Spectra were accumulated for 2.0 × 10⁵ fission events. Mass calibration was based on H⁺ and Na⁺ ions. All spectra were background-subtracted. The Bio-Gel P-2 purified sample was dissolved in small portions under slow rotation and a constant flow of nitrogen onto a nitrocellulose-covered aluminized polyester backing (21).

Direct Morgan-Elson Assay
The linkage type of the amino sugar was determined by the direct Morgan-Elson assay (22). In addition, the linkage of the amino sugar was proved by alkaline treatment (23).

RESULTS
Isolation of a Heterosaccharide—The glycan strands were split off from the isolated S-layer glycoprotein by hydrazinolysis (11, 12). The acetylated and reduced glycan strands (12) were desalted on a small Dowex 50 W × 5 H⁺ column. Then the desalted hydrazinolysate was separated on a TSK HW40 S column (Fig. 1). The carbohydrate-containing fractions were subsequently run on a reversed phase column (ProRPC HR16/10, C2-C8) (Fig. 2). Further purification of the carbohydrates was achieved by a Nucleosil NH₂ column (Fig. 3). In order to remove the low molecular weight compounds still remaining in the glycan fraction, a Bio-Gel P-2 column was used with water as eluent. Peak 2 contained the isolated heterosaccharide (Fig. 4). The fractions of this peak were pooled and run on an Aminex HPX 42 A column. Only one carbohydrate-containing peak was found (Fig. 5). The homogeneity of this fraction was tested in addition by thin layer chromatography (Table I) and with a Dionex PA1 column, from which the heterosaccharide eluted after 33.15 min (Fig. 6). The analysis of the oligosaccharides obtained after sequential splitting of O- and N-linked glycans (13) showed that only one N-linked glycan type existed in the isolated glycoprotein.

Chemical Composition—The chemical analysis of the hydroxylsates of the heterosaccharide revealed that it is composed of D-mannose, D-3-O-methylmannose (3-O-methylglucose), and D-N-acetylgalactosamine in a molar ratio of 3:2:1. The
alditol acetates of the two 3-O-methylhexoses had the same retention time on the Durabond capillary column. 3-O-methylmannose was identified in the heterosaccharide by NMR spectroscopy (see below). Both 3-O-methylhexoses were identified with gas-liquid chromatography after splitting off the methyl group with boron tribromide (18) as mannose and glucose. 3-O-methylmannose and 3-O-methylglucose were present in the intact glycoprotein in a molar ratio of 7:1. The demethylation procedure was performed with the intact glycoprotein but not with the isolated heterosaccharide, because the pure heterosaccharide was obtained in low amounts. All components of the glycan possess the d-configuration (see "Materials and Methods").

Anomeric Configuration—The anomeric configurations of the components of the glycan strand were determined by treatment of the purified heterosaccharide as well as the intact isolated glycoprotein with chromium trioxide (14). 3-O-methylhexoses (90%), mannose (95%), and galactosamine (96%) remained intact. These results show that the sugars occur in the α-configuration, which was also proved by NMR spectroscopy. The linkage between N-acetylgalactosamine and asparagine should also possess the α-configuration, because galactosamine was not destroyed with CrO₃ in the intact glycoprotein.
Partially methylated alditol acetates were obtained by gas-liquid chromatography (Table II) and the glycan was detected in peak IV. Peaks I–III contained no carbohydrate. Dashed line, sodium acetate gradient.

**Table II**

Methylation analysis of the isolated heterosaccharide

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<th>m/z</th>
<th>Relative intensities</th>
<th>%</th>
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<td>CH₃J</td>
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* Peaks obtained by gas-liquid chromatography after methylation of the isolated heterosaccharide.

**Selected fragments.**

**Linkage of the Glycan Components**

The linkage of the glycan components was determined by methylation analysis (15) of the isolated heterosaccharide. Three peaks (A–C) of partially methylated alditol acetates were obtained by gas-liquid chromatography (Table II). The characteristic fragments (m/z, Table II) and intensities of peak A are indicative of a 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl hexitol, while peak B corresponds to a 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl heptitol (16). Peak C indicates a 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl hexitol.

In order to distinguish whether peak A, B, or C corresponds to a 3-O-methylhexose, the methylation analysis was performed with CD₃J instead of CH₃J. The characteristic shift of m/z of the fragments (Table II) shows that peak A corresponds to a partially methylated mannose and peaks B and C to a partially methylated 3-O-methyl hexose.

Therefore, the mannose residues occur in a (1 → 2) linkage, while the 3-O-methylhexoses are (1 → 6) linked. Both sugars exist in the pyranosidic form, because the C-atom 4 was methylated, and the C-atom 5 was acetylated in each case. The 3-O-methyl hexose is the sugar residue at the nonreducing end of the glycan chain. The amounts of N-acetylgalactosamine in the glycoprotein was determined before and after oxidation with periodate. The amino sugar was completely recovered after the periodate treatment. That indicates that the remaining glycan strand should be linked to the C₂ or C₄ of N-acetylgalactosamine. The linkage could not be assigned with certainty by methylation analysis and NMR, because no peak of a methylated alditol derivative of galactosamine could be detected by gas-liquid chromatography after methylation, and no interpretable signal was obtained by NMR. The direct Morgan-Elson assay (22) with the isolated glycan was negative, while with chondrosine, a positive signal was obtained. For the Morgan-Elson test, the glycan obtained after hydrazinolysis was not reduced with NaBH₄. After incubation with ammonia solution (23), the amino sugar was completely recovered with an amino acid analyzer. Therefore, the remaining glycan strand should be linked to the C₆ of N-acetylgalactosamine.

In addition, the 1 → 6 linkage of the 3-O-methylmannose residues was confirmed by acetylation. After acetylation, two peaks were separated on an Aminex HPX 42A column. The first peak contained a pentasaccharide (elution time 9.20 min) consisting of mannose, N-acetylgalactosamine and 3-O-methylmannose in a molar ratio of 3:1:1.2. In the second peak...
preferentially. Gel containing fractions of peak I1 were found. Treatment with acetic acid anhydride cleaves preferentially $1 \rightarrow 6$ linkages with high efficiency. Therefore, the 3-O-methylmannose should be the glycan component at the nonreducing end of the heterosaccharide linked $1 \rightarrow 6$ to the second 3-O-mannose residue.

**Mass Spectrometry**—The positive ion plasma desorption mass spectrum (Fig. 7) of the pooled carbohydrate-containing fractions of peak II obtained after Bio-Gel P-2 separation revealed a sodiated molecular ion ($[M + Na]^+$) at $m/z$ 1084.82 with high abundance. The measured relative molecular mass (1061.83) is in good agreement ($\Delta m = -0.16 u, -0.015\%$) with the calculated value (1061.99) and consistent with the proposed structure in the reduced form based on the kind and ratios of the determined monosaccharides. No useful negative ion plasma desorption mass spectrum was obtainable. In addition to this dominating ion, abundant sodiated molecular ions at $m/z$ 1098.08 and 1140.38 were detected. The first ion corresponds to the hexasaccharide carrying a hydrazinyl substitution at the reducing end. The second ion carries an additional acetyl group. Both modifications are generated during the isolation and purification procedure. Further, a minor peak in the higher mass range was found at $m/z$ 1436.03 representing again a sodiated molecular ion. This ion is likely to correspond to the described hexasaccharide carrying two more O-methyl hexose units. The calculated average relative molecular mass of this compound in the reduced form is 1414.33. The relative molecular mass was determined to be 1413.04 showing a mass deviation of $-1.29 u$ ($-0.091\%$).

**NMR Spectroscopy**—A partial structure of the carbohydrate composed of three monosaccharide units could be elucidated by modern NMR techniques. By means of 1D and 2D TOCSY (24, 25) experiments, the proton resonances were assigned to the individual monosaccharide units (see Fig. 8). The propagation of the magnetization through the coupling network depends on the duration of the mixing time. We used a long mixing time to be sure that starting from the signals of anomeric protons all correlation signals of the corresponding residue were detected. The sequence of protons in each residue was deduced from a H, H-COSY spectrum. Information about the relative configuration of the carbon atoms in a sugar residue could be obtained by determination of the vicinal coupling constants ($J_{\text{ax,eq}} \approx 8$ Hz, $J_{\text{ax,ax}} \approx 0$ Hz, $J_{\text{eq,eq}} \approx 3$ Hz). From these configurations, the nature of sugar residues can be derived. Information about the relative configuration at the anomeric center can be obtained from the value of the $J_{\text{C,H}}$ of C-1 (axial, $J_{\text{ax}} \approx 160$ Hz; equatorial, $J_{\text{eq}} \approx 170$ Hz) (26). Thus, we established 3 α-mannopyranosyl residues.

Cross-peaks in an HMBC spectrum (Heteronuclear Multiple-Bond Connectivity; H-detected C,H correlation, optimized for small coupling constants) (27), between H-3 of manp-VI and manp-V to a carbon of an O-methyl group showed the existence of two 3-O-methyl-α-mannopyranose units. Information about the sequence of the oligosaccharide chain and the linkage sites was obtained by the scalar coupling between a carbon and a proton of the neighboring residues by means of an HMBC experiment and by measuring the dipolar couplings of neighboring anomeric protons and linkage site protons of different residues by ROESY experiments (28). We detected correlations between manp-VI (position 1) and manp-V (position 6) and between manp-V (position 1) and manp-IV (position 2), which established the partial structure 3-O-methyl-α-mannop- VI-1 → 6)-3-O-methyl-α-mannop- V-1 → 2)-α-mannp-IV within the oligosaccharide (see Fig. 9).

The $^1$H and $^13$C NMR spectrum exhibits signals characteristic of an acetyl group and, furthermore, in $^1$H NMR spectrum, of an NH proton.

$^13$C resonances were assigned by an HMBC (Heteronuclear Multiple-Quantum Coherence experiment; $^1$H-detected H,C correlation) (29) and an HMQC-TOCSY (30).

**DISCUSSION**

The glycan of the S-layer glycoprotein of the extremely thermophilic methanogen *M. fervidus* was chemically analyzed. Based on the chemical composition and other data mainly obtained by methylation analysis and NMR as well as mass spectrometry, a proposal of the glycan structure is depicted in Fig. 9. The isolated oligosaccharide is a hexasaccharide with N-acetylgalactosamine at the reducing end and 2 (1 → 6) linked O-methyl sugar residues at the nonreducing end. Between the O-methyl sugars and the amino sugar, 3 (1
→ 2) linked mannose residues are situated. We assume that in the intact glycoprotein the hexasaccharide should be N-glycosidically linked to asparagine via N-acetylglactosamine, because the isolated heterosaccharide had GaIN at the reducing end, and a glycopeptide having only left asparagine as single amino acid of equal molar amount as GaIN was obtained by subsequent enzymatic digestion of the peptide moiety with pepsin and proteinase K (data not shown). This type of N-glycosidic linkage has been exclusively found in the extremely halophilic archaea (31) so far. Twenty sequon structures Asn-X-Thr/Ser as potential N-glycosylation sites have been identified in the peptide sequence (5). About 10 of these sequon structures carry a hexasaccharide. This could be calculated from the total amount of galactosamine and sequon structures present in the intact glycoprotein (4, 5).

While in procaryotes, N- and O-glycosidically linked glycans are present in S-layer glycoproteins (1, 2), only one glycan type was obtained by different procedures (13) in the case of M. fervidus. In Halobacterium halobium, being somewhat related to the methanogens, three different glycan structures occur in the S-layer glycoprotein (1). Furthermore, in contrast to the halobacteria, no acidic groups are present, and not a transient O-methylation but a permanent O-methylation is found. However, two different types of nucleotide-activated oligosaccharides as potential precursors of the S-layer glycoprotein were isolated from cell extract of M. fervidus (6).

Based on the structural information from the intact S-layer glycoprotein, we assume that the second glycan precursor may be constituent of another glycoprotein, which builds up the flagella (32). In order to understand the further processing more precisely, we will perform in vitro studies with enzyme preparations. In halobacteria, the proteins of the flagella are also glycosylated (33).

The glycans of eubacterial and archaea glycoproteins exhibit a high degree of variation (1, 2, 34–36). No common basic structure could be detected as in N-linked eucaryotic glycophobins (37). While of the glycan of M. fervidus, an additional variant is added to the known glycan structures of procaryotic glycoproteins. While in eucaryotes, glycoprotein glycans fulfill several important functions (38), a correlation between a certain structure and function was proposed only for halobacterial S-layer glycoproteins (39, 40). Whether the glycan structure of the S-layer glycoprotein of M. fervidus is involved in the stabilization of surface proteins at high temperature or plays a role in cell aggregation in Methanothermus sociabilis, which possesses a similar peptide and carbohydrate composition (4, 5), remains an open question at the moment.

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