HALOBACTERIAL GLYCOPROTEIN SACCHARIDES CONTAIN COVALENTLY LINKED SULPHATE

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1. Introduction

The main component of *Halobacterium halobium* cell wall (envelope fraction) is a glycoprotein of M_r 200 000 analogous to the *Halobacterium salinarium* cell wall protein in [1].

Two types of saccharides are attached to this glycoprotein:

(i) About 35 threonine-linked di- or trisaccharides containing no amino sugar (oligosaccharides);
(ii) One asparagine-linked saccharide of much higher M_r containing amino sugars in addition to neutral hexoses (heterosaccharide) [1].

This glycoprotein has a form-giving or form-keeping function [2]: if grown in the presence of bacitracin, the rod-shaped halobacteria convert to regular spheres. This change is correlated with the loss of some carbohydrates within the glycoprotein [3].

Here we show that the oligosaccharides and the heterosaccharide of the cell wall glycoprotein contain covalently bound sulphate.

2. Materials and methods

2.1. *Halobacteria growth*

Halobacteria were grown in a complex medium under conditions in [4].

2.2. *Sulphate labelling*

For incorporation of $^{35}SO_4^{2-}$, cells were grown in a sulphate-depleted complex medium, made up with NaCl analytical grade (Merck) and substituting $MgSO_4$ by $MgCl_2$ (Merck). Carrier-free $^{35}SO_4^{2-}$ (Amersham) was added to give a radioactivity of 1–3 μCi/ml. Growth was at 38°C in 100 ml Erlenmeyer flasks containing 30 ml culture, which were shaken at 150 rotations/min.

2.3. *Isolation of the glycoproteins*

Halobacterial cell envelope glycoprotein was isolated according to [1]. This procedure also led to the isolation of glycoprotein from halobacterial spheres, which were obtained by growing the cells in the presence of 5–10 μg bacitracin/ml (Sigma) (*H. halobium*) and 50 μg/ml (*H. salinarium*).

2.4. *Analytical methods*

2.4.1. Amino acid and amino sugar analyses

Amino acids and amino sugars were analyzed in an automatic amino acid analyzer (Biotronic) on a Durrum 6 A column combined with fluorometric detection of the amino acids after reaction with *o*-phthalic aldehyde [5].

Neutral sugars were analyzed by gas–liquid chromatography after reduction and conversion to the corresponding alditol acetates [6] on a 6 ft Supelco 3% SP-2340 column with a linear temperature gradient from 200–240°C at 2°C/min.

2.4.2. Determination of sulphate

The samples were hydrolyzed at 110°C overnight in constant boiling HCl. After evaporation the samples were passed through a small column of Dowex AG 50 W H^+ using water as eluant. The effluent was evaporated and used for sulphate determination according to [7]. Protein was estimated by amino acid analyses of aliquots taken before the Dowex step.

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2.5. *Isolation of $^{35}SO_4^{2-}$-glycopeptides*

2.5.1. Pronase digestion

For digestion, $^{35}SO_4^{2-}$-labelled envelopes or isolated glycoproteins were incubated with pronase (Sigma) (1–2%, w/w) for 15 h at 39°C and further 5 h at 56°C. To remove pronase and peptide materials, the samples were chromatographed on Dowex AG 50 W H^+ ion-exchange resin in water. The eluant containing 90–95% of the radioactivity was lyophilized or evaporated to dryness.

2.5.2. Separation of $^{35}SO_4^{2-}$-glycopeptides

The glycopeptides were resuspended in 0.1 M pyridine–acetate buffer (pH 5.5) and chromatographed on Sephadex G-25 or G-50 in the same buffer. For separation by high-performance liquid chromatography (HPLC) the glycopeptide mixtures were resuspended in water and chromatographed on a silica gel dimethyl amino column (Nucleosil, Macherey-Nagel, Düren) on a Waters high-performance liquid chromatograph (the conditions are under fig.3).

2.6. *Hydrazinolysis*

Hydrazinolysis was done essentially as in [8]. Anhydrous hydrazin (Baker) and dry hydrazinium sulfate (Baker) was used.

3. Results and discussion

Halobacterium salinarium and *H. halobium* cell wall glycoproteins comigrate on SDS–polyacrylamide gels. In addition, fingerprint analyses as in [9] demonstrate identical cleavage patterns of the *H. salinarium* and *H. halobium* cell wall glycoproteins. Therefore the data for the *H. salinarium* glycoprotein in [1,2] were used in this study on *H. halobium* glycoprotein.

3.1. *Cell wall glycoprotein contains covalently linked sulphate*

Halobacteria grown in a sulphate-depleted medium readily incorporate $^{35}SO_4^{2-}$. SDS gel electrophoresis of ^{35}S-labelled cell homogenates display mainly 3 radioactive high M_r bands as detected by fluorography (fig.1). After acid hydrolysis of these substances eluted from the SDS gel the total radioactivity is recovered as [^{35}S]sulphate as judged by thin-layer chromatography on polyethylene imine cellulose plates. Obviously these halobacterial species do not reduce sulphate.

The most slowly migrating radioactive component comigrates with the cell wall glycoprotein on SDS gels. Identity of this $^{35}SO_4^{2-}$-labelled material and of cell wall glycoprotein was established by fingerprint analysis according to [9]: Periodate–Schiff base (PAS)-staining of halobacterial cell envelopes from $^{35}SO_4^{2-}$-labelled cells after SDS gel electrophoresis

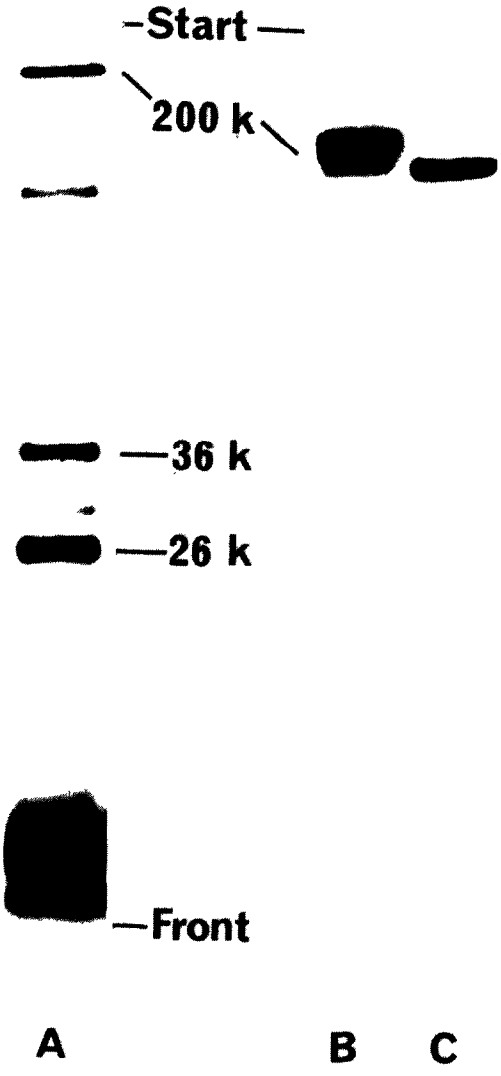

Fig.1. $^{35}SO_4^{2-}$-Labelling pattern of *H. halobium* cell lysate and of cell wall glycoproteins. Fluorographs of SDS–polyacrylamide gel electrophoresis according to [10,11]. Gels contained 12% (A) and 6% (B,C) acrylamide. (A) Cell lysate after $^{35}SO_4^{2-}$-labelling as in section 2. (B) Isolated $^{35}SO_4^{2-}$-labelled cell wall glycoprotein from rod-shaped cells. (C) $^{35}SO_4^{2-}$-labelled isolated cell wall glycoprotein from spherical cells grown in the presence of bacitracin.

exhibits one single high M_r band [1] (fig.2A). After degradation by limited *Staphylococcus aureus* protease treatment this glycoprotein yields the PAS-staining pattern of fig.2B. Fig.2C,D are autoradiographes of (A,B) and exhibit bands identical with the PAS-stained patterns. The autoradiograph of (B) (fig.2D) in addition shows a regular pattern of nearly equidistant bands. The explanation for this additional characteristic banding will be given in the following.

The sulphate content of purified glycoprotein was determined as in section 2 and found to be 20 μg sulphate/mg protein. Based on M_r 200 000, ~40 sulphate residues are linked to 1 glyco protein molecule.

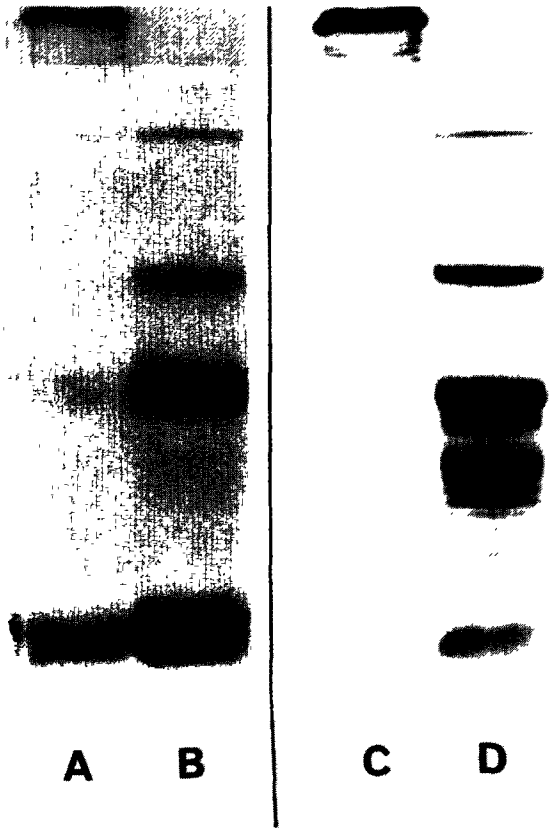

Fig.2. Fingerprint analysis of $^{35}SO_4^{2-}$-labelled cell envelopes according to [9]. Glycopeptides were visualized by periodate Schiff base (PAS) staining according to [12] (A,B) and by radioautography (C,D) after electrophoresis on 12% SDS–polyacrylamide gels. (A) $^{35}SO_4^{2-}$-labelled *H. halobium* cell envelopes, PAS-stain; (B) sample (A) after treatment with *S. aureus* protease (25 μg/ml, 37°C, 10 min, according to [9]), PAS stain; (C,D) autoradiographs of (A) and (B), respectively.

3.2. *Sulphate is linked to both the oligosaccharides and the heterosaccharide*

To investigate the binding sites of sulphate, isolated $^{35}SO_4^{2-}$-labelled glycoprotein was digested with pronase. Sulphate containing peptides were purified and separated by HPLC as in section 2 (fig.3A). The fractions containing radioactivity were analyzed for their sugar contents. Each of the peaks (a–i) contains glucose and galactose, but no amino sugars. In contrast, peak (z), eluting at much higher ionic strength contains amino sugars and mainly galactose. Based on the analytical data of [1] the radioactive peaks (a–i) therefore represent oligosaccharide containing peptides while peak (z) is a heterosaccharide containing peptide fraction. These results suggest the

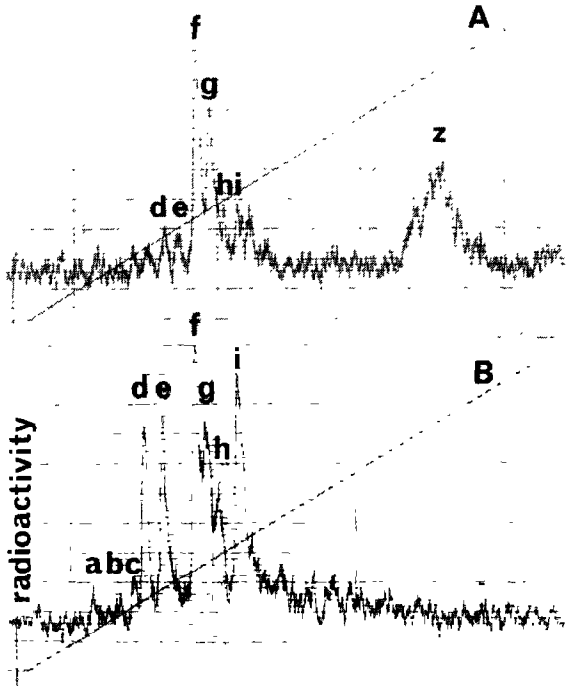

Fig.3. Separation by HPLC of pronase derived peptides from $^{35}SO_4^{2-}$-labelled cell wall glycoproteins. The column was 300 × 4 mm, packed with Nucleosil 10 N (Macherey-Nagel). After injection of the samples the column was washed with 0.1 M ammonium formiate buffer (pH 4.0) for 15 min at a flow rate of 1.4 ml/min. Thereafter a 45 min gradient was applied from 0.1–1.2 M of the same buffer and at the same flow rate. (A) Peptides from glycoprotein isolated from rod-shaped cells; (B) from spherical cells. Radioactivity was detected by a Berthold scintillation counter equipped with a 100 μl flow cell.

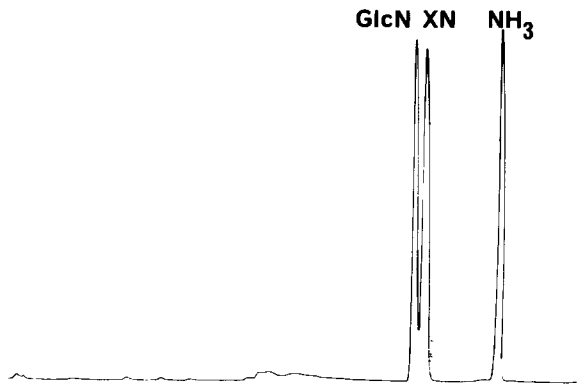

Fig.4. Amino sugar and amino acid analysis of peak (z) (cf. fig.3A) after hydrazinolysis and gel filtration on Sephadex G-50.

presence of sugar sulphates. This was confirmed for the heterosaccharide containing fraction: hydrazinolysis of peak (z) material yielded a radioactive heterosaccharide, free of amino acids (fig.4). Thus an amino acid side chain as the sulphate binding site is excluded.

Analyses of the $^{35}SO_4^{2-}$-heterosaccharide peptide fraction on SDS–polyacrylamide gels and subsequent fluorography gave a surprising result: the material of peak (z) (fig.3A) did not migrate as a single band, but displays up to 20 nearly equidistant bands (fig.5B). This pattern is not due to an artifact, as may be concluded from the following data:

(i) It occurs independently of the protease used for the digest (e.g., pronase, *S. aureus* protease, trypsin);
(ii) It is not influenced by varying the growth conditions of the halobacteria (e.g., different $[SO_4^{2-}]$ in the medium, different growth rates of the cells achieved by different aeration rates);
(iii) Hydrazinolysis yields a product with a similar behaviour.

3.3. *Identification of a sulphated glycoprotein lacking the heterosaccharide*

Spherical cells grown in the presence of bacitracin also incorporate $^{35}SO_4^{2-}$ into their cell wall glycoprotein. Glycoprotein from such spheres has the same amino acid composition as normal glycoprotein. However, it shows a lower app. M_r on SDS gels (fig.1C). This material does not contain any amino sugars, indicating the absence of the heterosaccharide. In addition, analyses of $^{35}SO_4^{2-}$-labelled peptides (pronase digest) by HPLC revealed that it lacks the peak (z) found in normal glycopeptides (fig.3B). Accordingly, on SDS–polyacrylamide gel fluorographs the multitude of equidistant bands due to the $^{35}SO_4^{2-}$-heterosaccharide peptide fraction is also missing (fig.5A). On the other hand, most of the peaks representing O-linked oligosaccharides are present in this digest.

The absence of the heterosaccharide in the glycoprotein isolated from spherical cells suggests that it is the heterosaccharide moiety which is involved in keeping the rod form of halobacteria.

The multiple bands displayed by the $^{35}SO_4^{2-}$-heterosaccharide on fluorographs on SDS gels could be explained by the assumption that the 'heterosaccharide' is a repetitive sequence of a sulphated saccharide unit. In this case the different equidistant bands would represent different chain lengths of a sequence similar to the animal glycosaminoglycans.

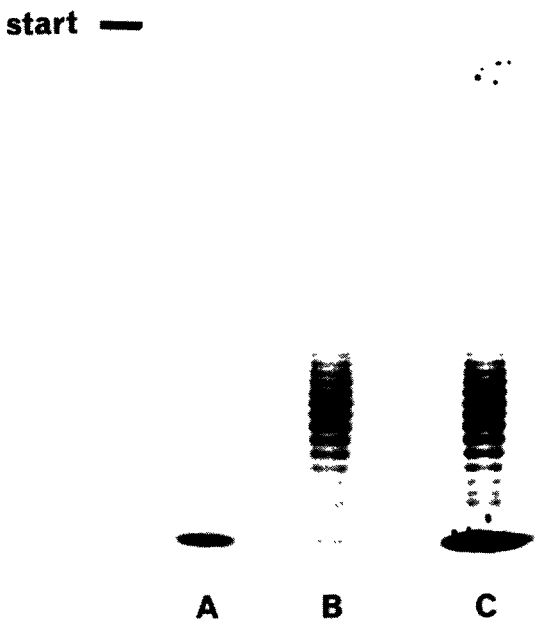

Fig.5. Analysis of pronase-derived $^{35}SO_4^{2-}$-labelled peptides on SDS–polyacrylamide gel electrophoresis according to [10,11]: (A) $^{35}SO_4^{2-}$-glycopeptides derived from glycoprotein of spherical cells; (B) peak (z) material (cf. fig.3A); (C) $^{35}SO_4^{2-}$-glycopeptides derived from glycoprotein of rod-shaped cells.

Acknowledgements

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