SULPHATION OF A REPETITIVE SACCHARIDE IN HALOBACTERIAL CELL WALL GLYCOPROTEIN

Occurrence of a sulphated lipid-linked precursor

F. WIELAND, J. LECHNER, G. BERNHARDT and M. SUMPER

Institut für Biochemie, Genetik und Microbiologie, Lehrstuhl Biochemie I, Universität Regensburg, Universitätsstraße 31, 8400 Regensburg, FRG

1. Introduction

In [1] we reported that the halobacterial cell wall glycoprotein, studied in detail [2], contains at least two species of sulphated saccharides: amino-sugar free oligosaccharides of low M_r and one high M_r saccharide (HM_r-saccharide) containing mainly amino-sugars and galactose (heterosaccharide).

The gel electrophoretic pattern of this sulphated 'heterosaccharide' gave rise to the assumption that it represents a repetitive sequence of a sulphated unit rather than a heterosaccharide structure. The function of this glycoconjugate in determining the rod form of the bacteria has been suggested [1,3].

Here we prove the suggested repetitive structure of this saccharide. Furthermore, a sulphated lipid-linked precursor of this glycoconjugate is described indicating a biosynthetic pathway different from that known for glycosaminoglycans.

2. Materials and methods

2.1. Halobacterial growth

Halobacterium halobium was grown in a complex medium under conditions in [4].

2.2. Isolation of aminosugar containing sulphated HM_r-glycopeptides

The $^{35}SO_4^{2-}$-glycopeptides derived from pronase digestion of $^{35}SO_4^{2-}$-labelled cell wall glycoprotein were isolated and separated as in [1].

2.3. Isolation of gel electrophoretically homogeneous $^{32}SO_4^{2-}$-HM_r glycopeptides

Isolation was performed by gel electrophoresis according to [5] on gels containing 12% (w/v) acrylamide (Serva, Heidelberg) in the presence of 6 M urea. Gels were 0.5 mm diam. and 40 cm long. 5 × 10^6 cpm of isolated HM_r-glycopeptides were subjected to electrophoresis for 4 h at 800–1000 V. After autoradiography, single gel strips were cut out according to the pattern on the film, homogenized and the radioactive material was eluted. After concentration, the glycopeptides were purified by gel filtration on Sephadex G-25 in 0.1 M pyridine–acetate buffer (pH 5.5) and dried by evaporation.

2.4. Partial acid hydrolysis

The salt free samples were dissolved in water and mixed with 1 vol. 0.5 N HCl. Incubation was for 15 min in a boiling water bath. Thereafter the samples were dried by lyophilisation.

2.5. Hydrazinolysis and nitrous acid deamination

Hydrazinolysis was as in [1]. The dried product was redissolved in 0.5 M Na-acetate buffer (pH 3.5) containing 50 mg $NaNO_2$/ml. After 60 min at room temperature the product was purified on Sephadex G-25 in pyridine–acetate buffer 0.1 M (pH 5.5) and dried by evaporation. Subsequently the samples were analysed by high-voltage thin-layer electrophoresis (for details see section 2.7).

2.6. *Isolation of lipid-linked $^{35}SO_4^{2-}$-saccharides*

Synthetic medium: Chemicals were of the highest purity grade available (Merck). One liter contained: 240 mg each of the L-amino acids Pro, Met, Arg, His, Thr, Ser, Val, Lys, Gly, Cys, Phe, Leu, Fleu, Ala, Glu, Gln, Asp, Asn and 120 mg each of Tyr and Phe; 250 g NaCl, 20 g $MgCl_2$, 2 g KCl, 500 mg K_2HPO_4, 2.5 mg $MnCl_2$, 100 mg $CaCl_2$, 5 mg $FeCl_3$. After sterilization the following vitamins were added: pyridoxal 0.5 mg; pantothenic acid 0.5 mg; thiamine 0.025 mg; nicotinic acid 0.025 mg; riboflavin 0.025 mg; *p*-aminobenzoic acid 0.005 mg; biotin 0.005 mg; vitamin B_{12} 0.0005 mg. The pH was adjusted to 6.5; 40 ml synthetic medium was innoculated with one clone of halobacteria from an agar culture and shaken at 38°C and 100 rev./min in 100 ml Erlenmeyer flasks.

Cells grown for 4 days in synthetic medium were concentrated 20-fold in sulphate free basal salts (250 g NaCl, 20 $MgCl_2$, 2 g KCl and 1 l H_2O) and incubated for 1 h at 38°C under illumination. Then, to 1 ml cell suspension 40 μg ethidium bromide (Sigma) was added together with 80–100 μCi $Na_2$$^{35}SO_4$ (carrier free, Amersham). After 6 h under illumination with gentle stirring at 38°C the cells were centrifuged, washed with basal salt solution (250 g NaCl, 20 g $MgSO_4$, 2 g KCl and 1 l H_2O) and lyzed by stirring in 500 μl 50 mM Tris buffer (pH 7.5) containing 5 μg DNase (Boehringer, Mannheim) and 10 mM $MgCl_2$. The lyzate was diluted with 10 ml 50 mM Tris buffer (pH 7.5) and centrifuged for 2 h at 39 000 rev./min in a Beckman 50 Ti rotor. The pellet (membrane fraction) was resuspended in 300 μl H_2O and extracted twice with 7 ml chloroform/methanol = 3/2 and twice with 6 ml chloroform/methanol/water = 10/10/3. Then the residue was dried and extracted with 3 ml propanol/water = 1/1.

2.7. *Analytical methods*

High-voltage electrophoresis was performed on polygram 400 (Machery and Nagel, Düren) at 2200 V for 60 min in formic acid/acetic acid/water = 20/80/900 (pH 2.0). SDS–polyacrylamide gel electrophoresis was according to [6], fluorography according to [7]. Amino-sugar and neutral sugar determinations were as in [1]. Uronic acids were determined according to [8] and as their alditol acetates by gas liquid chromatography under [1] after reduction, lactonization and reduction with $NaBH_4$ (Merck, Darmstadt) according to [9]. Sulphate was determined after hydrolysis of the samples with 6 N HCl for 2 h at 100°C according to [10,11].

3. Results and discussion

3.1. *The amino-sugar containing $^{35}SO_4^{2-}$-saccharide fraction represents a repeating unit structure*

The amino sugar containing $^{35}SO_4^{2-}$-glycopeptide fraction is excluded on a Bio-Gel P10 column, indicating an M_r of (equivalent to globular proteins) >20 000. On gel electrophoresis in the presence or absence of SDS it displays a highly regular pattern of up to 15 bands (fig.1A) [1]. On the other hand this fraction gives rise to a single spot on high-voltage thin-layer electrophoresis (fig.4A, lane a) indicating a constant mass to charge ratio for all of the glycopeptides seen on the gel electrophoretic pattern. In order to check a possible structural relationship between the single homogeneous glycopeptides, 3 different glycopeptides far distant from each other were isolated from a gel as indicated with arrows in fig.1A. These glycopeptides were subjected to a 'fingerprint' analysis by partial acid hydrolysis and subsequent high-voltage electrophoresis. Fig.1B shows the resulting fluorogram: both the total glycopeptide mixture (lane a) and the different individual glycopeptides produced identical patterns (lanes b–d). From these data we conclude that this group of sulphated saccharides is made up of a repeating unit. Determinations of sulphate according to [10,11] as well as different sugar analyses (anthrone
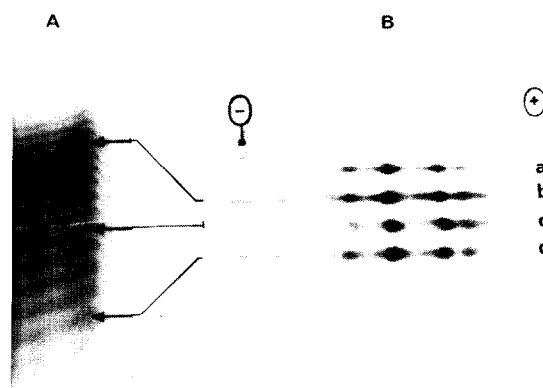

Fig.1. (A) Separation of $^{35}SO_4^{2-}$-labelled HM_r-glycopeptides by polyacrylamide gel electrophoresis in the presence of urea according to [5]. After autoradiography the material at the positions marked with arrows was eluted from the gel. (B) High-voltage thin-layer electrophoresis of the isolated homogeneous HM_r-glycopeptides after partial acid hydrolysis: total mixture of HM_r-glycopeptides (a) and isolated $^{35}SO_4^{2-}$-glycopeptides according to the arrows in fig.1A (b–d, respectively). For details see section 2.

method) [13], gas—liquid chromatography as in [1] and measurement of uronic acids according to [8] resulted in the following molar composition of the saccharides: 1 gal, 1 glcNH$_2$, 1 galNH$_2$, 2 galUA and 2 sulphate residues.

3.2. A sulphated precursor of the cell wall glycoprotein

Halobacteria were pulse labelled with $^{35}SO_4^{2-}$ and the lyzate was analyzed by SDS—polyacrylamide gel electrophoresis. Preceeding the formation of detectable radioactive cell wall glycoprotein a highly regular pattern appears consisting of up to 15 bands (fig.2a). Protease treatment did not affect this pattern. Furthermore, this pattern of sulphated bands is produced even in the presence of ethidium bromide in concentrations completely inhibitory for protein biosynthesis (fig.2d).
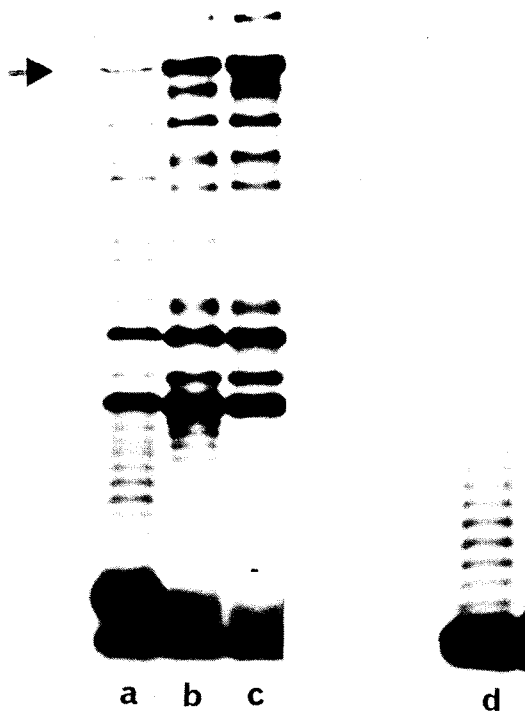
Therefore the material giving rise to this characteristic pattern does not contain protein. In a chase experiment, this radioactivity disappeared with a concomitant increase in radioactivity of the glycoprotein suggesting a precursor role of these $^{35}SO_4^{2-}$-labelled bands (lanes b,c). Therefore this material was isolated and further analyzed.
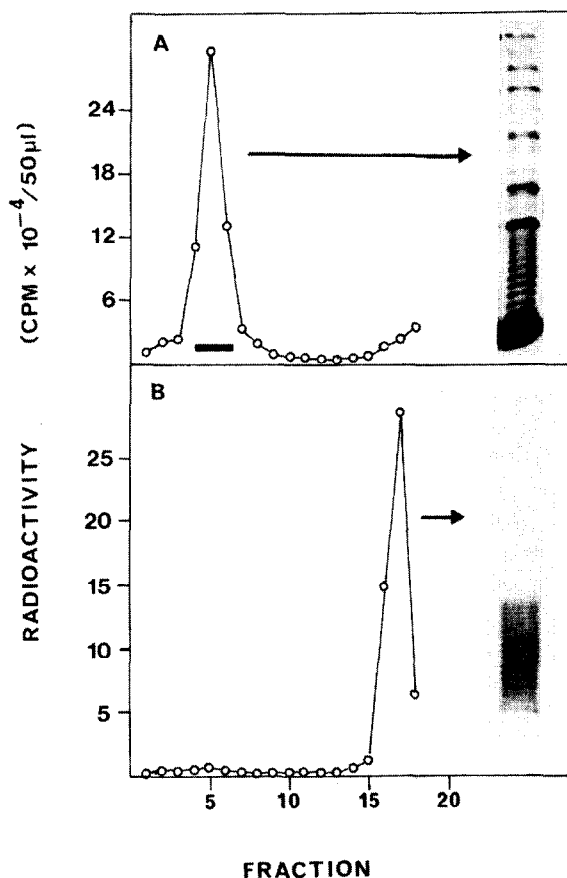

Fig.2. In vivo $^{35}SO_4^{2-}$ pulse-chase labelling experiments with halobacteria. Halobacteria grown in a sulphate-depleted complex medium were concentrated 15-fold and pulse-labelled with $^{35}SO_4^{2-}$ (100 μCi/ml, carrier free, Amersham). Fluorogram of a 12% (w/v) SDS—polyacrylamide gel according to [6]. (a) Total lyzate after pulse-labelling for 30 min; (b) lyzate after 60 min chase (performed by dilution with 2 vol. complex medium containing sulphate); (c) 120 min chase; (d) pulse labelling in the presence of ethidium bromide (40 μg/ml). The arrow markes the position of the cell wall glycoprotein.

FRACTION

Fig.3. The $^{35}SO_4^{2-}$-precursor saccharide is a membrane-bound component. Cell lyzates were centrifuged at 5°C for 2 h at 39 000 rev./min in 50 mM Tris—HCl buffer (pH 7.5), 1.0 M NaCl in a Beckman 50 Ti rotor. The pellets (crude membrane fractions) were suspended in the above buffer and loaded on sucrose density gradients (10—50% (w/v) sucrose in 50 mM Tris—HCl buffer (pH 7.5), 1.0 M NaCl) and centrifuged for 15 h at 10°C and 39 000 rev./min in a Beckman SW-40 rotor. Fractions were collected across the gradient from the bottom of the tubes. (A) Crude membrane fraction after pulse-labelling of halobacteria with $^{35}SO_4^{2-}$. (B) Unlabelled crude membrane fraction mixed with $^{35}SO_4^{2-}$-HM_r-glycopeptides. The bar markes the position of the cell membrane fraction. Inserts show fluorograms of SDS—polyacrylamide gels of the corresponding peak fractions.

Fig.4. Comparison by high-voltage thin-layer electrophoresis of $^{35}SO_4^{2-}$-HM_r-glycopeptides and $^{35}SO_4^{2-}$-lipid-linked saccharides after different fragmentation procedures. (A) Untreated $^{35}SO_4^{2-}$-HM_r-glycopeptides (a) and untreated $^{35}SO_4^{2-}$-lipid-linked saccharides (b); samples (a) and (b) after partial acid hydrolysis (c) and (d), respectively. (B) Hydrazinolysis and nitrous acid deamination of $^{35}SO_4^{2-}$-HM_r-glycopeptides (a), of $^{35}SO_4^{2-}$-lipid-linked saccharides (b) and of a mixture of both samples (c). Electrophoresis and fragmentation condition are given in section 2.

3.3. The sulphated precursor is lipid-linked

The pulse-labelled material exhibiting the characteristic banding on SDS-gels (fig.2a) is firmly associated with the cell membrane: after fractionation of a cell lyzate by sucrose density gradient centrifugation this material is quantitatively recovered in the membrane fraction (fig.3A). As a control, $^{35}SO_4^{2-}$-glycopeptides derived from pronase digestion of the cell wall glycoprotein were added to a crude membrane fraction. As shown in fig.3B these glycopeptides do not copurify with the membrane. Extraction of the membrane fraction twice with chloroform/methanol = 3/2 and twice with chloroform/methanol/water = 10/10/3 yields a membrane pellet still containing the $^{35}SO_4^{2-}$-precursor. Solubilization is achieved by treatment with propanol/water = 1/1. This solubility in a solvent more hydrophilic than the solvents usually used for polyprenyl—sugar compounds is easily explained by the presence of negative charges introduced by sulphate.

3.4. The lipid-linked sulphated material is a precursor of the repeating unit saccharide of the cell wall glycoprotein

The $^{35}SO_4^{2-}$-repeating unit fraction isolated after pronase digestion of the $^{35}SO_4^{2-}$-labelled cell-wall glycoprotein as well as the isolated $^{35}SO_4^{2-}$-lipid fraction were subjected to 'fingerprint' analysis: (i) by partial acid hydrolysis and (ii) by hydrazinolysis followed by nitrous acid deamination and subsequent high-voltage thin-layer electrophoresis on cellulose plates. Fig.4 shows the results: untreated lipid-linked sulphated material does not migrate at all due to its lipid character (A, lane b). In contrast, the isolated $^{35}SO_4^{2-}$-glycopeptides migrate essentially as a single spot (A, lane a). However, mild acid hydrolysis produces identical fragmentation patterns from both preparations (A, lanes c,d). The same result is obtained by hydrazinolysis followed by nitrous acid deamination (fig.4B). Taken together, these results show that the halobacterial cell wall glycoprotein contains a sulphated repeating unit saccharide which is synthesized on and transferred from a lipid carrier. Remarkably, the sulphate residues are detectable already in the lipid-linked precursor at all degrees of polymerization. In the biosynthesis of a glycosaminoglycan-like structure this seems to be unusual since, for example, in the synthesis of heparin, glycosidic polymerisation occurs step by step on a protein bound linker saccharide and the sulphate residues are introduced only after glycosidic polymerisation is finished [14,15].

Acknowledgements

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