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Halobacterial glycoprotein biosynthesis

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Contents

I. Introduction

In contrast to procaryotic organisms, the majority of cell surface carbohydrates of eucaryotic cells is present in glycoproteins. Mescher et al. [1–3] were probably the first authors to demonstrate the occurrence of a true glycoprotein on the cell surface of a procaryotic organism, namely on the cell surface of *Halobacterium salinarium*. Phylogenetically, halobacteria belong to the archaebacteria, the third kingdom of life besides eubacteria and eukaryotes. There are only a few other reports of procaryotic glycoproteins [4,5]. However, more recently there has been growing evidence that the cell surface layers of other Gram-negative archaebacteria (such as the Thermoacidophiles) may contain true glycoproteins as well (for review see Ref. 6): solubilized preparations of their cell surface layers yield PAS-positive protein species, but no detailed structural data of the carbohydrates involved are available.

Since procaryotes obviously lack the organelles

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typically involved in eucaryotic glycoprotein synthesis, the discovery of a procaryotic glycoprotein has stimulated further work on its structure and particularly on its biosynthesis. This review will briefly summarize our present knowledge on halobacterial glycoprotein structure and biosynthesis.

Halobacteria exhibit the typical Gram-negative archaebacterial cell envelope profile. The cells lack the rigid murein sacculus and an outer membrane typical of Gram-negative eubacteria. Instead, halobacteria are surrounded by a single layer of hexagonally arranged protein subunits [7–12]. X-ray diffraction studies as well as electron microscopy of thin sections were interpreted to demonstrate the existence of two protein layers outside the cytoplasmic membrane, separated by a 'periplasmatic space' [13,14]. However, chemical analysis of the halobacterial surface layer as well as comparison with surface layers of other archaebacteria favour the interpretation that the halobacterial surface layer is composed of a single glycoprotein species, which is very tightly joined to the cytoplasmic membrane [6,15].

During the initial studies, this procaryotic glycoprotein was isolated from cell envelopes of *Halobacterium salinarium* and was partially characterized. Its apparant molecular mass was estimated to be 200 kDa, with a carbohydrate content of 10–12%. Information regarding the number, composition, and type of carbohydrate-protein linkages was obtained by isolation and chemical characterization of the glycopeptides derived from degradation of the cell surface glycoprotein with trypsin and pronase. From the data obtained, it was concluded that the intact glycoprotein has a single N-linked complex-type oligosaccharide, 22 to 24 O-linked disaccharides and 12 to 14 O-linked trisaccharides per molecule [2]. More recent investigations have revealed further structural details of this glycoprotein and have corrected many of the original views of its structure.

II. Halobacterial glycoproteins: cell surface glycoprotein and flagellins

A significant feature of the cell surface glycoprotein is its extreme acidic nature. In chemical terms, this is explained by the high degree of sulfation of some of the oligosaccharide chains: up to 50 mol sulfate are bound in ester linkages per mol of cell surface glycoprotein [16].

This observation greatly simplified structural and biosynthetic studies, since incubation of halobacteria with [^{35}S]sulfate selectively labels the cell surface glycoprotein as well as its biosynthetic precursors, allowing their detection even in a crude cell extract. This selectivity of labeling is due to the fact, that halobacteria are unable to reduce sulfate. Fig. 1A shows the result of such a labeling experiment. A crude extract from [^{35}S]sulfate pulse-labeled halobacteria was separated by SDS-polyacrylamide gel electrophoresis and radioactive components were visualized by fluorography. In addition to the sulfated cell surface glycoprotein and low molecular weight components (sulfated precursors), a set of heterogenous sulfated proteins is observed with molecular masses of 25–36 kDa. Recently, these sulfated proteins were characterized as additional glycoproteins and identified as the halobacterial flagellins [17]. The unique properties of halobacterial flagella were recently described in Ref. 18.

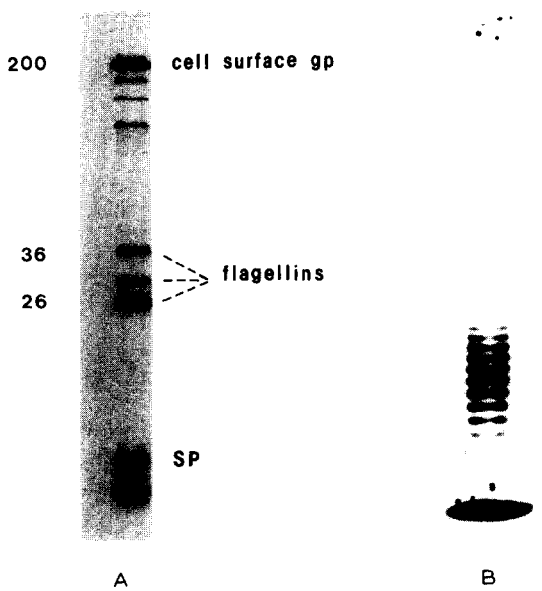


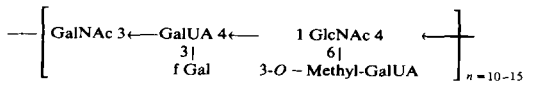
Fig. 1. Fluorogram of SDS-polyacrylamide gels loaded with a crude cell extract from $^{35}SO_4^{2-}$-labeled halobacteria (A) and with homogenous $^{35}SO_4^{2-}$-labeled cell surface glycoprotein after exhaustive digestion with pronase (B).

III. Structures of the saccharides linked to the cell surface glycoprotein

Exhaustive pronase digestion of purified [^{35}S] sulfate-labeled halobacterial cell surface glycoprotein and subsequent separation by permeation chromatography on Bio-Gel P-10 results in three fractions of glycopeptides [19]: within the void volume of the column ^{35}S-labeled material elutes, which contains both neutral sugars and the amino sugars glucosamine and galactosamine in a 1 : 1 stoichiometry. In addition, this material contains galacturonic acid and its unusual derivative 3-*O*-methylgalacturonic acid [20]. This sulfated glycopeptide represents the socalled 'heterosaccharide' originally described in Ref. 2. The glycopeptide fraction of intermediate molecular weight contains only the neutral sugars glucose and galactose but is free of any [^{35}S]sulfate radioactivity. The low molecular weight glycopeptide fraction again contains [^{35}S]sulfate radioactivity together with glucose, glucuronic acid and some iduronic acid. Thus, the cell wall glycoprotein contains two types of sulfated saccharides in addition to neutral saccharides.

III-A. Sulfated high molecular weight saccharide

Analysis of the ^{35}S-labeled glycopeptide fraction of high molecular weight on a polyacrylamide gel (with or without SDS) gave a most surprising result [21]: the material did not migrate in a single band but displayed a highly regular pattern of up to 20 bands (Fig. 1B). This result offered strong evidence that the published structure of a heterosaccharide is incorrect. Rather, this material was likely to represent different chain lengths of a sulfated repeating unit saccharide. Subsequent detailed structural characterization confirmed this assumption: the building block of this repetitive sequence is a pentasaccharide, being composed of 1 galactose, 1 GalNAc, 1 glcNAc, 1 GalUA and 1 3-*O*-methyl-GalUA [19,20]. According to recent permethylation data, the following structure could be assigned to the pentasaccharide building block [22]:



Remarkable features of this repeating unit saccharide are the occurrence of 3-*O*-methylgalacturonic acid and of furanosidic galactose, both of which are linked peripherally to the sugar backbone. Each of the building blocks in addition contains two moles of sulfate. One of the sulfate residues was found to be linked to the 4-position of GalNAc [22]; the position of the remaining sulfate residue could not yet be determined. The repeating unit saccharide is directly linked to the protein in a *N*-glycosidic bond (i.e., without any core region as is found in animal proteoglycans). This mode of attachment implies the presence of a novel type of *N*-glycosidic linkage, namely asparaginyl-*N*-acetylgalactosamine. The existence of this linkage unit was proved by the isolation and chemical characterization of asparaginyl-*N*-acetylgalactosamine [20]. This finding was unexpected as all eucaryotic *N*-glycosidic linkages known so far contained asparaginyl-*N*-acetylglucosamine [23]. The sulfated repeating unit saccharide is present only once per cell surface glycoprotein molecule.

III-B. Sulfated low molecular weight saccharides

The sulfated low molecular weight saccharides consist of Glc, GlcUA and iduronic acid [19,24]. The ratio of glucuronic acid to iduronic acid is about 3 : 1. Iduronic acid, a main constituent of animal proteoglycans has been found only once in procaryotes [25]. The complete structure of these saccharides was established by permethylation analysis: the hexuronic acids are linearly arranged in 1–4 linkages and are linked to the protein via another hitherto unknown type of *N*-glycosidic linkage unit, namely asparaginylglucose [26,27]. Each hexuronic acid is sulfated, most probably at position 3 [28]. Since each molecule of the cell surface glycoprotein possesses about ten oligosaccharide chains of this type it is not surprising that some structural heterogeneity is found within this oligosaccharide family. In particular, the peripheral position of the linear hexuronic acid chain is variable, being either occupied by a hexuronic acid or a glucose residue.

III-C. Nonsulfated glycopeptides

The nonsulfated glycopeptide fraction of intermediate molecular weight contains only the neu-

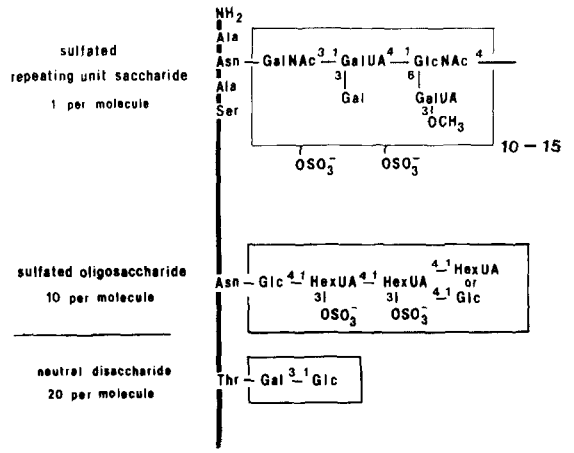


Fig. 2. Structure of the sulfated cell surface glycoprotein of halobacteria.

tral sugars glucose and galactose. These sugars are linked to the peptide as disaccharides via *O*-glycosidic bonds to threonine residues [2]. About 20 disaccharides occur in a highly clustered arrangement within the polypeptide chain thus producing a relatively large glycopeptide even after exhaustive pronase digestion. Permethylation analysis of the disaccharides established a Glc 1 → 3 Gal linkage between the sugar residues [22].

Fig. 2 summarizes the structural data obtained from the various saccharides of the cell surface glycoprotein.

IV. Structure of the saccharides linked to flagellins

Halobacterial flagella consist of a set of (three) related glycoproteins with sulfated oligosaccharides. To investigate the chemical nature of the covalently bound sulfate residues, isolated flagella were digested exhaustively with subtilisin and pronase. All of the sulfate residues were recovered in the glycopeptide fraction. These sulfated glycopeptides were found to be indistinguishable from the sulfated oligosaccharides found in the cell surface glycoprotein. Furthermore, these saccharides are attached to the flagellins by the same unusual linkage unit asparaginylglucose [17], i.e., these glycoconjugates are of the type

Glc 1 → 4 HexUA 1 → 4 HexUA 1 → 4 Glc-Asn and
HexUA 1 → 4 HexUA 1 → 4 HexUA 1 → 4 Glc-Asn

with HexUA being either glucuronic acid or to a lesser extent iduronic acid. Additional evidence that this type of oligosaccharide is common to all halobacterial glycoproteins comes from the fact that these sulfated glycoproteins share the same pool of sulfated oligosaccharide precursors (see below).

V. Amino acid sequences of the *N*-glycosidic linkage units

To characterize the surroundings of the novel *N*-glycosidic linkage units asparaginylglucose and asparaginyl-*N*-acetylgalactosamine, cell surface glycoprotein as well as flagellins were digested to yield the corresponding glycopeptides. Some of the individual glycopeptides could be purified by reverse phase HPLC. The results of peptide sequencing are summarized in Table I. All the amino acid sequences surrounding the *N*-glycosidic linkages fit the acceptor sequence Asn-X-Thr(Ser)-common to all eucaryotic *N*-glycosidically linked glycoconjugates [29]. Remarkably, the sulfated repeating unit saccharide of the cell surface glycoprotein is linked to an asparagine residue positioned next to the glycoprotein's N-terminus at position 2 of the amino acid sequence [20].

In order to get more detailed protein sequence information from halobacterial glycoproteins, a halobacterial gene bank was established in the high level expression vector pI-N-III-A constructed by Inouye and coworkers [31]. A set of 40 000 clones was initially screened with a rabbit polyclonal antibody to purified flagellin I, resulting in the isolation of a single immunopositive clone. Any expression of DNA cloned in this expression vector results in a fusion protein consisting of the amino-terminal *Escherichia coli* prolipoprotein amino acids followed by insert-coded amino acids. DNA sequence analysis of the insert of the immunopositive clone confirmed the isolation of a halobacterial flagellin gene fragment, since the derived amino acid sequence exactly matched the sequences of isolated flagellin peptides (Ref. 32, Gerl. L., Paul, G., Wieland, F. and Sumper, M., unpublished results). Unfortunately, the insert only codes for 181 amino acids and lacks the coding sequence for the N-terminal region of the flagellin. Therefore the decision

TABLE I

AMINO ACID SEQUENCES SURROUNDING THE PROTEIN-CARBOHYDRATE LINKAGE UNITS OF HALOBACTERIAL GLYCOPROTEINS

Linkage unit	
Asn-GalNAc	NH_2-Ala-Asn-Ala-Ser-Asp | CHO (cell surface glycoprotein)
Asn-Glc	Val-Asn-Ser-Ser-Gly | CHO (cell surface glycoprotein)
	Ile-Asn-Leu-Thr-Lys (flagellin) | CHO

whether the isolated gene fragment codes for flagellin I, II or III cannot be made yet, since all three flagellins are structurally related and immunologically crossreactive [17]. Nonetheless, the protein sequence deduced so far includes three possible glycosylation sites, one of which has already been verified by glycopeptide sequence data.

VI. Biosynthesis of the sulfated repeating unit saccharide

Growth of halobacteria in the presence of the antibiotic bacitracin was found to result in a reduced level of glycosylation of the cell wall glycoprotein [34]. Subsequent studies revealed that it is only the sulfated repeating unit saccharide which is completely absent in the glycoprotein of treated bacteria, whereas no change in the level of sulfated oligosaccharides occurs [16]. This specific bacitracin effect suggests operation of a cyclic pathway involving lipid pyrophosphate-linked saccharides in the biosynthesis of the repeating unit saccharide. This assumption could be verified by pulse labeling experiments with [^{35}S]sulfate and subsequent analysis of cell lysates on SDS-polyacrylamide gels. Preceding the formation of detectable amounts of radioactive glycoprotein, a highly regular pattern of labeled material appears on the fluorogram consisting of up to 15 bands. This labeling pattern is produced even under conditions of inhibited protein biosynthesis (Fig. 3). The labeled material copurifies with the halobacterial cell membrane and has a strong amphiphilic character as is typical of lipid-linked oligosaccharides. Its relationship to the sulfated repeating unit saccharide of the glycoprotein was investigated by comparing the fragmentation patterns obtained by partial acid hydrolysis. From these results identity of the saccharide part derived from the glycolipid and the glycoprotein was confirmed [21]. The inhibitory action of bacitracin indicated that the sulfated repeating unit saccharide may be linked via a pyrophosphate bridge to the lipid moiety. No data exist concerning the exact nature of the lipid anchor, i.e., whether it is an undecaprenyl type as has been suggested previously [35] or a dolichol derivative which has been shown to be involved in the biosynthesis of the sulfated oligosaccharides (see below). To summarize the information obtained so far: about 15 bands are found on the gel. They are thought to

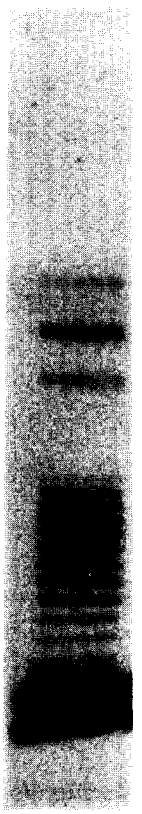


Fig. 3. Fluorogram of a 12% SDS-polyacrylamide gel loaded with a cell lysate from halobacteria $^{35}SO_4^{2-}$-labeled (15 min) under conditions of completely arrested protein synthesis (50 μg/ml ethidium bromide).

TABLE II

NUCLEOTIDE SEQUENCE OF A HALOBACTERIAL INSERT CLONED IN THE EXPRESSION VECTOR pI-N-III-A AND CODING FOR 181 AMINO ACIDS OF A HALOBACTERIAL FLAGELLIN

Boxed amino acid sequences were confirmed by peptide sequencing. Glycosylation sites are underlined.

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GATC GGC ACA CTC ATC GTG TTC ATC GCG ATG GTG CTG GTC GCC GCG ATC
 ile gly thr leu ile val phe ile ala met val leu val ala ala ile

 GCC GCC GGC GTC CTC ATC AAC ACT GCC GGC TAC CTC CAA TCC AAG GGG
 ala ala gly val leu ile asn thr ala gly tyr leu gln ser lys gly

 TCC GCA ACT GGT GAG GAA GCC TCC GCA CAG GTC TCC AAC CGC ATC AAC
 ser ala thr gly glu glu ala ser ala gln val ser asn arg ile asn

 ATC GTC TCC GCG TAC GGC AAC GTG GAC ACG TCT GGC TCA ACC GAG GTA
 ile val ser ala tyr gly asn val asp thr ser gly ser thr glu val

 GTC AAT TAC GCG AAC CTG ACG GTG CGC CAG GCC GCT GGG GCT GAC AAC
 val asn tyr ala asn leu thr val arg gln ala ala gly ala asp asn

 ATC AAC CTC AGC AAA TCC ACG ATC CAG TGG ATC GGC CCG GAC ACC GCC
 ile asn leu ser lys ser thr ile gln trp ile gly pro asp thr ala

 ACT ACC TTG ACC TAC GAC GGG ACT ACT GCC GAC GCC GAG AAC TTC ACC
 thr thr leu thr tyr asp gly thr thr ala asp ala glu asn phe thr

 ACG AAT TCG ATT AAG GGC GAC AAC GCG GAC GTG CTG GTT GAT CAG TCC
 thr asn ser ile lys gly asp asn ala asp val leu val asp gln ser

 GAC CGC ATC GAG ATC GTC ATG GAC GCG GCC GAG ATC ACC ACC AAT GGA
 asp arg ile glu ile val met asp ala ala glu ile thr thr asn gly

 CTG AAG GCT GGC GAA GAG GTC CAG CTG ACA GTG ACC ACC CAG TAC GGC
 leu lys ala gly glu glu val gln leu thr val thr thr gln tyr gly

 TCG AAA ACC ACC TAC TGG GCG AAC GTT CCT GAG TCG CTC AAG GAC AAA
 ser lys thr thr tyr trp ala asn val pro glu ser leu lys asp lys

 AAC GCA GTC ACG CTA TAA CAC ACA CGC TCA TGT TCG AGT TCA TCA CTG
 asn ala val thr leu stop

 ACG AGG ACG AGC GCG GTC AAG TGG GGA TC
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represent about 15 chains differing in length of the lipid-linked repeating unit saccharide, the distance between two adjacent bands being most probably due to one repeating unit. Remarkably, the sulfate residues are detectable already in these lipid-linked precursors at all degrees of polymerization. From the little data available, it seems plausible to assume that the polymerization of the repeating pentasaccharide unit occurs by a mechanism analogous to that worked out for Salmonella O-antigen biosynthesis [36–38].

VII. Biosynthesis of sulfated oligosaccharides

In vivo pulse labeling with [^{35}S]sulfate and subsequent analysis of the halobacterial cell lysates by SDS gel electrophoresis yields fluorographs as shown in Fig. 4. After the pulse, the main labeled product is a low molecular weight component (lower arrow in Fig. 4). This material exhibits a high turnover rate: during the chase all of its incorporated radioactivity is transferred to the halobacterial glycoproteins. Consequently, this

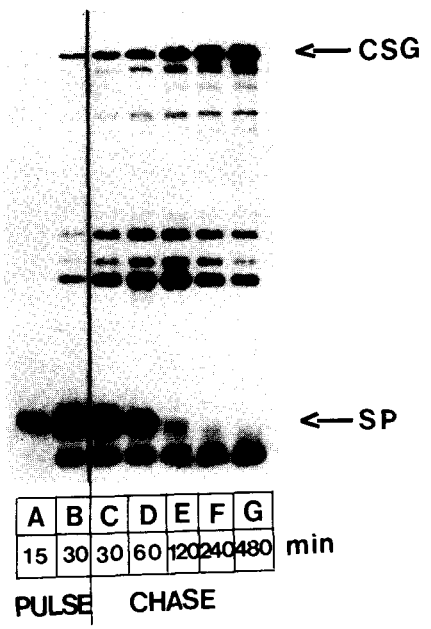

Fig. 4. $^{35}SO_4^{2-}$ pulse-chase labeling of halobacteria. A halobacterial cell suspension was pulse labeled with $^{35}SO_4^{2-}$ and aliquots were taken after 15 and 30 min. The chase was performed after 30 min by addition of unlabeled sulfate for the times indicated. Cells were lysed by hypoosmosis and submitted to electrophoresis on a 12% SDS-polyacrylamide gel. The figure shows a fluorogram of the gel. CSG, cell surface glycoprotein, SP sulfated precursor.

sulfated material is a likely candidate for a biosynthetic precursor of sulfated saccharides (sulfated precursors). Sulfated precursors copurified with the halobacterial membrane fraction and could be extracted into $CHCl_3 : CH_3OH : H_2O$ (10 : 10 : 3) mixtures, a typical extraction protocol for lipid-linked oligosaccharides. After several purification procedures, including ion exchange chromatography and HPLC on silica gel a family of closely related sulfated glycolipids was obtained. They all contained lipid, phosphate, sulfate, glucose and hexuronic acids. Their lipid moiety was characterized by ^{1}H-NMR spectroscopy, EI-mass spectroscopy as well as by its resistance to acid hydrolysis as a C_{60} polyprenylphosphate of the eucaryotic dolichol type [26]. Surprisingly, the oligosaccharides were found to be attached via a monophosphate bridge. The isolated sulfated precursors differed from each other solely with respect to their oligosaccharide moieties. Detailed chemical analysis of the individual lipid-linked oligosaccharides revealed that they all matched the following general formula

$$R \rightarrow 4\ \text{HexUA}\ 1 \rightarrow \left[4\ \text{HexUA}\ 1 \rightarrow\right]_{1\text{-}2} 4\ \text{glucose}\ 1 \rightarrow \text{P - lipid}$$

R = H or glucose or 3-*O*-methylglucose

Hex UA = glucuronic acid or (about 1/3) iduronic acid

With a single exception, all the carbohydrate structures of sulfated precursors are compatible with those found in the sulfated oligosaccharides of the cell surface glycoprotein and the flagellins. The full structure of the sulfated precursors is given in Fig. 5.

Some of the dolichylphosphate oligosaccharides contained a peripherally bound 3-*O*-methylglucose, although no 3-*O*-methylglucose could be detected either in the cell surface glycoprotein or in the flagellins [39]. The lack of 3-*O*-methylglucose in the acceptor proteins indicates that this residue represents a transient modification of the lipid oligosaccharides.

Unexpectedly, transfer to the protein of the sulfated oligosaccharides turned out to be strictly dependent on this transient methylation, since inhibitors of *S*-adenosylmethionine-dependent methylations (such as the combination of homocysteine and adenosine [40]) strongly depressed in vivo production of sulfated glycoproteins. This inhibition of glycoprotein synthesis was not due to an inhibition of protein biosynthesis in general, as control incubations with [^{35}S]methionine in the presence and absence of the methylation inhibitor both yielded very similar labeling patterns. In addition, synthesis of unmethylated sulfated precursors was not markedly affected by the methylation inhibitor. Therefore, essentially two possible functions of the transient methylation of the dolichyl-linked oligosaccharides are conceivable:

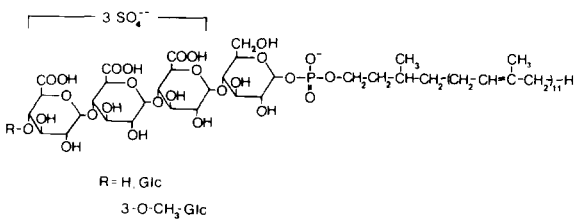

Fig. 5. Structure of the sulfated precursors.

since dolichol derivatives do not translocate spontaneously across the lipid bilayer [41] the methylation could represent an obligatory step for translocation of the lipid oligosaccharide. Alternatively, the 3-*O*-methylglucose could serve a function similar to that attributed to the glucose residues on the lipid-linked Glc_3 Man_5 $GlcNac_2$ or Glc_1Man_5 $GlcNac_2$ [42,43] involved in eucaryotic glycoprotein synthesis, namely to facilitate the transfer of the oligosaccharide to protein [44–51]. It is not known whether the 3-*O*-methylglucose residue is removed from the oligosaccharide at the lipid-linked level or at the protein-linked level. However, there is an experimental observation favoring the latter possibility. The synthetic hexapeptide Tyr-Asn-Leu-Thr-Ser-Val containing a sequon sequence is able to compete in vivo for the sulfated oligosaccharides (see below). Analysis of the oligosaccharide structure transferred to this peptide indicated that the terminal 3-*O*-methylglucose was still present (Lechner, J. and Sumper, M., unpublished results). It remains to be established whether the 'trimming' reaction is a demethylation or a demethylglucosylation. In this context it is of interest that in the fungus *Mucor rouxii* a methylation reaction was recently found in the processing of high mannose-type oligosaccharides [67].

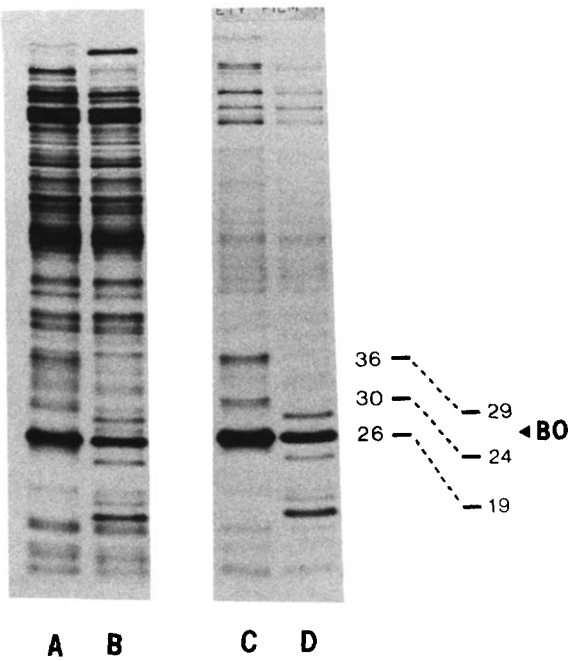

Fig. 6. In vivo pulse labeling of halobacteria with [^{35}S] methionine in the absence (A) and presence (B) of EDTA in the incubation medium. Cell lysates (A, B) were applied to a 12% SDS-polyacrylamide gel and labeled proteins were visualized by fluorography. C and D: sedimentable components of lysates A and B obtained by ultracentrifugation. The numbers indicate the apparent molecular masses (in kDa) of flagellin I, II and III and of their derivatives (19, 24, 29) produced in the presence of EDTA. Flagellin I (26 kDa) comigrates on the gel with the membrane protein bacterio-opsin (BO).

VIII. Cellular location of halobacterial glycoprotein synthesis

The involvement of lipid-linked saccharides suggests that, in halobacteria too, glycoprotein synthesis is a membrane-mediated process. If transfer of the saccharide to the protein occurs in a compartment equivalent to the luminal site of the eucaryotic rough endoplasmic reticulum, glycosylation in halobacteria should occur at the extracellular surface of the cell membrane. Indeed, some observations support this idea:

(1) The antibiotic bacitracin inhibits glycosylations by binding to lipid pyrophosphates [53,54]. In halobacteria, bacitracin was shown to be unable to enter the cell [34], yet it selectively inhibits transfer to the protein of the repeating unit saccharide [21]. This observation suggests that this reaction takes place at the cell surface. Transfer to the protein of sulfated oligosaccharides remains unaffected by bacitracin, in agreement with the finding that a lipidmonophosphate oligosaccharide serves as precursor rather than a pyrophosphate.

(2) In vitro glycosylation with synthetic peptides has shown that the Asn-X-Thr(Ser)- sequence is sufficient for oligosaccharide transfer [50,51, 55–61]. Since this typical acceptor sequence is also present in halobacterial glycoproteins, a synthetic hexapeptide (Tyr-Asn-Leu-Thr-Ser-Val) was tried in vivo as an artificial exogenous acceptor for sulfated oligosaccharides [39]. In this experiment, halobacteria were incubated with or without hexapeptide in the presence of [^{35}S]sulfate. Dependent on the presence of hexapeptide, sulfated glycopeptide material appeared in the incubation medium, supporting the hypothesis of an ex-

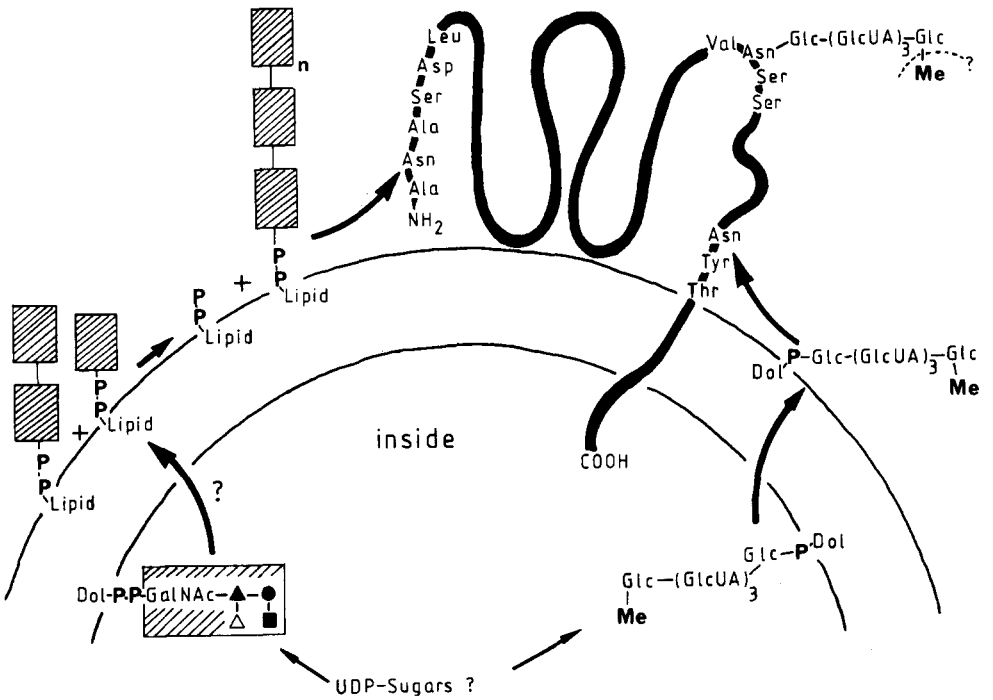

Fig. 7. Biosynthesis of the *N*-glycosidic linkage units Asn-GalNAc and Asn-Glc of the halobacterial cell surface glycoprotein. A lipid pyrophosphate and a dolichylmonophosphate serve as the saccharide carriers. Me, methyl; ▲, GalUA; ●, GlcNAc; △, Gal; ■, 3-*O*-Methyl-GalUA.

tracellular location of the active site of the oligosaccharyltransferase.

(3) Addition of EDTA to the incubation medium of halobacteria influences in a very specific manner the spectrum of newly synthesized proteins [62]. Whereas synthesis of nearly all intracellular proteins remains unaffected (Fig. 6A and B), a drastic alteration is observed with respect to glycoprotein synthesis: the flagellins I, II and III are no longer produced; instead immunocrossreactive components with apparant mol. masses of 19, 23 and 29 kDa appear in the gel patterns (Fig. 6C and D). Exactly the same components are obtained from the intact flagellins simply by chemical deglycosylation with anhydrous hydrogen fluoride. Therefore, the altered synthetic pattern probably reflects specific inhibition of the oligosaccharyltransferase by exogenously added EDTA and independently supports the extracellular location of this enzyme.

XI. Concluding remarks

The cell surface glycoprotein of halobacteria shows some structural analogies to the proteoglycan-collagen complexes of the extracellular matrix of animal tissues [63]: it contains a sulfated repeating unit saccharide reminiscent of the structure of animal glycosamino-glycans like keratan sulfate, although the halobacterial repeating unit structure turned out to be more complex. Furthermore, the cell surface glycoprotein contains about 20 *O*-glycosidically linked disaccharides, Glc 1 → 3 Gal which are similar to the neutral disaccharides Glc 1 → 2 Gal found in collagen. These structural parallels might support speculations articulated by Lamport and co-workers, that archaebacteria contain the original 'pro-eucaryotic' type of glycoprotein from which an evolutionary connection exists with the collagen/proteoglycan extracellular matrices of animals and the extensins of plants [64,65].

With respect to the protein-carbohydrate linkage unit, the halobacterial glycoproteins exhibit novel features as yet not known from eucaryotic glycoproteins: two different *N*-glycosidic linkages are synthesized within the same polypeptide chain. A single Asn-GalNAc linkage unit is created using a lipid pyrophosphate-linked precursor and a lipid

monophosphate-linked precursor is used to create the Asn-Glc linkage units. These pathways are summarized in the scheme of Fig. 7. All halobacterial *N*-glycosidic asparagines occur in the common acceptor sequence Asn-X-Thr(Ser)-, further supporting the idea that the hydroxyl amino acid is involved in the catalytic mechanism of the glycosyltransfer reaction [66]. However, the unique situation of two different types of *N*-glycosidic bonds within the same halobacterial cell surface glycoprotein requires additional recognition signals for the discrimination of the individual glycosylation sites of the polypeptide chain. Hopefully, sequencing of the gene of the cell surface glycoprotein currently under way in our laboratory will provide more detailed information.

The precursors of sulfated oligosaccharides of halobacterial glycoproteins exhibit unusual features as well. (1) The sulfated oligosaccharides are built up on and transferred from a dolicholmonophosphate rather than a pyrophosphate. (2) The lipid-linked oligosaccharide is fully sulfated (and epimerized) before its transfer to the polypeptide rather than becoming sulfated at the polypeptide level as in animal glycosaminoglycan biosynthesis. Clearly, halobacteria are equipped with a number of enzymatic activities found also in eucaryotic glycoprotein synthesis. However, the biosynthetic machinery of halobacterial glycoprotein synthesis is not distributed among specialized subcellular compartments. Perhaps as a consequence, all chemical modifications of the saccharides are performed exclusively at the lipid-linked level. The lipid carrier in halobacteria turned out to be of the eucaryotic type rather than a procaryotic undecaprenol.

At present, no information is available concerning the mechanism and site of biosynthesis of the neutral disaccharides *O*-glycosidically linked to the cell surface glycoprotein. A further unresolved problem is the functional role of the transient modification of the lipid monophosphate oligosaccharide with a peripherally bound 3-*O*-methylglucose.

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