



Protein glycosylation in *Haloferax volcanii*: partial characterization of a 98-kDa glycoprotein

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Abstract

The plasma membrane of *Haloferax volcanii* contains several glycoproteins, including a 98-kDa species. Using lectin-based chromatography, the glycoprotein was isolated and partially characterized. Sequence comparison, based on antibody binding as well as one-dimensional peptide maps show that the 98-kDa glycoprotein is distinct from the S-layer glycoprotein, the major glycoprotein in *H. volcanii*. The 98-kDa glycoprotein can be further distinguished from the S-layer glycoprotein on the basis of membrane attachment. Unlike the S-layer glycoprotein, the 98-kDa glycoprotein is not associated with the membrane in a Mg^{2+} -dependent manner. Both proteins, however, apparently rely on a similar mechanism of glycosylation, since neither was affected by treatment with bacitracin or tunicamycin, agents known to interfere with protein glycosylation in other species. Finally, the pattern of glycosylation of the 98-kDa glycoprotein is not shared by a 95-kDa glycoprotein of the related *Haloferax mediterranei* strain. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Archaeon; Glycoprotein; Membrane; Concanavalin A; Bacitracin; *Haloferax volcanii*

1. Introduction

Since the first description of a glycosylated prokaryotic protein, the surface (S) layer glycoprotein of the extreme halophilic archaea *Halobacterium salinarum* [1], glycosylated S-layer and flagellar proteins have been detected in numerous archaeal species [2,3]. As in Eukarya, archaeal N-glycosylation occurs at asparagine residues of the Asn-X-Ser/Thr consensus motif and involves similar saccharide subunits [2–4]. Archaea contain dolichyl-linked oligosaccharides, similar to those involved in eukaryotic protein glycosylation [5–7], while many of the enzymes of eukaryal protein glycosylation find archaeal counterparts [5,8–10]. Furthermore, the topologies of protein glycosylation in Eukarya and Archaea are apparently similar. Eukaryal protein glycosylation occurs on the luminal face of the endoplasmic reticular membrane [11]. Topologically, this corresponds to the external face of the archaeal plasma membrane, the proposed site of archaeal protein glycosylation [4]. Protein glycosylation in Eukarya and Archaea may, therefore, share a common evolutionary origin [5].

As such, further understanding of archaeal protein glycosylation could elucidate the parallel process in eukaryal cells.

Apart from S-layer glycoproteins and flagellins, only a limited number of other glycosylated archaeal proteins have been reported [2,3], such as the membrane-associated glycoprotein of *Thermoplasma acidophilum* [12] and a series of glycoproteins from isolated membranes of *Sulfolobus acidocaldarius* [13]. Glycosylation of archaeal extracytoplasmic proteins may, however, be more widespread than currently thought. Recently, the presence of several glycoproteins in the plasma membrane of *Haloferax volcanii* was shown [14]. In the following report, partial characterization of the most prominent of these, the 98-kDa *H. volcanii* glycoprotein, is presented.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), DNase, N-acetylglucosamine (GlcNAc), periodic acid, phenylmethylsulfonyl fluoride, Schiff reagent, trichloroacetic acid (TCA), Triton X-100, and urea were from Sigma (St. Louis, MO, USA).

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Molecular mass markers were from Bio-Rad (Hercules, CA, USA) or Novex (San Diego, CA, USA). Endoproteinase Glu-C from *Staphylococcus aureus* V8 came from Boehringer-Mannheim (Mannheim, Germany). Tryptone and yeast extract were from Biolife (Milan, Italy). Concanavalin A (ConA)-Sephacrose was from Pharmacia (Uppsala, Sweden). Centricon-30 concentrating units came from Amicon (Beverly, MA, USA). Redivue [^{35}S] cell labelling mixture (> 1000 Ci/mmol) came from Amersham (Buckingham, UK).

2.2. Strains and growth conditions

H. volcanii DS2 was obtained from the American Type Culture Collection and grown aerobically at 40°C as previously described [15]. *Haloflex mediterranei* R4 was obtained from Richard Shand (Northern Arizona University, Flagstaff, AZ, USA) and grown aerobically at 40°C in *H. mediterranei* medium [16].

2.3. Purification of 98-kDa glycoprotein

Cells (2 l) were harvested ($8000 \times g$, 30 min) and resuspended in water to which DNase (~ 120 U) was added. The suspension was ultracentrifuged (45 000 rpm, 60 min, 60Ti rotor, Beckman L7-80 ultracentrifuge) and pellets were resuspended in lectin buffer (1% Triton X-100, 1 mM CaCl_2 , 1 mM MnCl_2 , 50 mM Tris-HCl, pH 7.9). The samples were then incubated with 1 ml ConA-Sephacrose beads first pretreated in lectin buffer containing 1 mg ml^{-1} BSA and then pre-eluted with 20 mM GlcNAc before being returned to lectin buffer. After gentle rocking (2 h, RT), the beads were washed with lectin buffer (20 ml), lectin buffer containing 1 M NaCl (20 ml) and then eluted with 100 mM GlcNAc.

2.4. Periodic acid-Schiff reagent staining

Periodic acid-Schiff reagent (PAS) staining was performed as described previously [17]. SDS-PAGE gels were incubated in 7.5% acetic acid (30 min, room temperature (RT)), transferred to 0.2% periodate (1 h, 4°C), then to Schiff reagent (1 h, 4°C). The stained gels were returned to 7.5% acetic acid (30 min, RT) and extensively washed in water.

2.5. One-dimensional peptide mapping

One-dimensional peptide maps were prepared essentially as previously described [18]: after Coomassie staining and destaining, gel regions containing the S-layer and 98-kDa glycoproteins were excised and incubated with buffer P (1 mM EDTA, 0.1% SDS, 125 mM Tris-HCl, pH 6.8) (10 ml, 30 min). Individual gel slices were then added to the wells of a second gel and overlaid with 10 μl of buffer P containing 20% glycerol. Finally, 10 μl of a solution of

buffer P containing 10% glycerol and 0–0.5 μg of *S. aureus* V8 endoproteinase Glu-C were added to the wells. SDS-PAGE was performed as normal except that the current was shut off for 30 min when the bromphenol blue dye front approached the bottom of the stacking gel.

2.6. Bacitracin and tunicamycin treatment

Cells in minimal medium [15] were grown in the absence or presence of bacitracin (25 $\mu\text{g ml}^{-1}$) or tunicamycin (50 $\mu\text{g ml}^{-1}$) at 40°C, overnight. The cells were then ^{35}S -radio-labelled (15 $\mu\text{Ci ml}^{-1}$, 90 min), collected ($8000 \times g$, 30 min, RT) and resuspended in lectin buffer. The samples were then incubated with ConA-Sephacrose beads as above and examined by SDS-PAGE and fluorography.

3. Results

3.1. Isolation of the 98-kDa glycoprotein

Earlier studies portrayed 190-, 150-, 98-, 58- and 54-kDa glycosylated proteins intimately associated with the plasma membrane of the halophilic archaeon *H. volcanii* [14]. While it was shown that the 190-kDa glycoprotein corresponds to the S-layer glycoprotein, the identities of the other *H. volcanii* glycoproteins remain unknown. In the present study, the 98-kDa glycoprotein was addressed.

To isolate the 98-kDa glycoprotein, membrane proteins were solubilized from isolated *H. volcanii* membranes and incubated with ConA-Sephacrose beads. Subsequent elution of the lectin-bound glycoproteins with GlcNAc and examination by SDS-PAGE and Coomassie staining revealed the capture of similar amounts of the 190-kDa S-layer glycoprotein and the 98-kDa glycoprotein (Fig. 1, lane 2). Control experiments revealed that these proteins do not originate from the lectin beads, while the specific interaction of these glycoproteins with ConA has been previously shown [14]. To confirm the glycosylated nature of the lectin-captured proteins, the GlcNAc-eluted material was subjected to PAS staining. As the prominent glycoprotein in *H. volcanii*, the S-layer glycoprotein was far more strongly stained by PAS in intact cells than the 98-kDa glycoprotein (lane 3), as previously reported [14]. However, following enrichment by ConA chromatography, PAS staining revealed similarly intense labelling of both the 190-kDa S-layer glycoprotein and the 98-kDa glycoprotein (lane 4).

3.2. The 98-kDa glycoprotein is distinct from the S-layer glycoprotein

It has been previously reported that antibodies raised against the intact S-layer glycoprotein (or sequences derived therefrom) are unable to label the 98-kDa glycoprotein in *H. volcanii* cells [14]. To confirm that the failure to

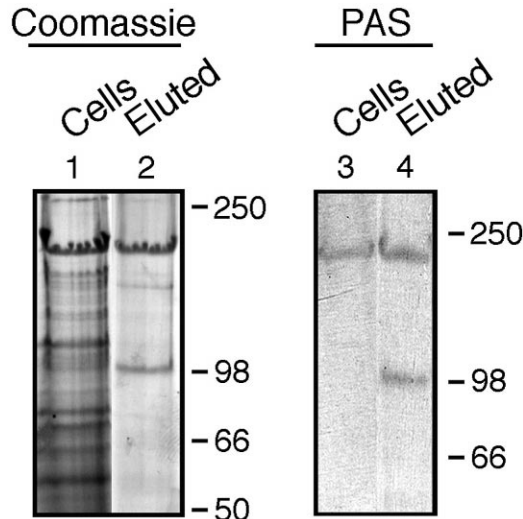


Fig. 1. The 98-kDa glycoprotein can be isolated from *H. volcanii* membranes by ConA-based chromatography. As described in Section 2, extracted proteins of *H. volcanii* membranes were incubated with ConA-Sepharose beads and eluted with 100 mM GlcNAc. The total cellular protein content (lanes 1 and 3) and ConA-bound proteins (lanes 2 and 4) were analysed by Coomassie staining (lanes 1 and 2) and PAS staining (lanes 3 and 4). Molecular mass markers are shown on the right of each panel.

label was not due to insufficient levels of the 98-kDa species being present in the native membranes, similar quantities of the S-layer and 98-kDa glycoproteins, as eluted from the ConA column, was transferred to nitrocellulose and probed with antibodies raised against the N-terminal 13 amino acid residues of the S-layer glycoprotein [19]. Again, the antibodies failed to recognize the 98-kDa glycoprotein (Fig. 2A), confirming that the S-layer glycopro-

tein and the 98-kDa glycoprotein do not share the same N-terminal sequence. Furthermore, polyclonal serum raised against the intact S-layer glycoprotein also failed to label the 98-kDa glycoprotein (not shown).

To further show that the 98-kDa glycoprotein does not correspond to a precursor or degradation product of the 190-kDa S-layer glycoprotein, one-dimensional peptide maps were generated from the S-layer and 98-kDa glycoproteins using *S. aureus* V8 endoproteinase Glu-C. The presence of a high proportion of acidic amino acids in haloarchaeal proteins [20] favours the use of the V8 endoproteinase for the generation of peptide maps, since the protease cleaves proteins primarily after glutamic acid residues [21]. As judged by intensities of Coomassie staining, SDS-PAGE gel slices containing similar amounts of the two glycoproteins were applied to the wells of a second gel in the absence or presence of protease (Fig. 2B). In the absence of added endoproteinase Glu-C, minor fragmentation of the S-layer and 98-kDa glycoproteins was detected (lane 1 and 2, respectively). Substantial 98-kDa glycoprotein digestion was achieved upon exposure to increasing amounts of endoproteinase (lanes 3, 5 and 7). While marked digestion of the S-layer glycoprotein was also observed, a significant fraction of the protein remained undigested (lanes 4, 6 and 8). In the presence of 0.05 μg protease, obvious differences in the digestion patterns of the two glycoproteins were apparent (lanes 3 and 4). At a higher endoproteinase Glu-C level (0.1 μg), the digestion patterns appeared more similar in terms of the number of peptide fragments, yet apart from a prominent ~ 20 -kDa band, these patterns were not coincident (lanes 5 and 6). At higher endoproteinase levels (0.5 μg), only a limited number of peptide bands were observed, but these

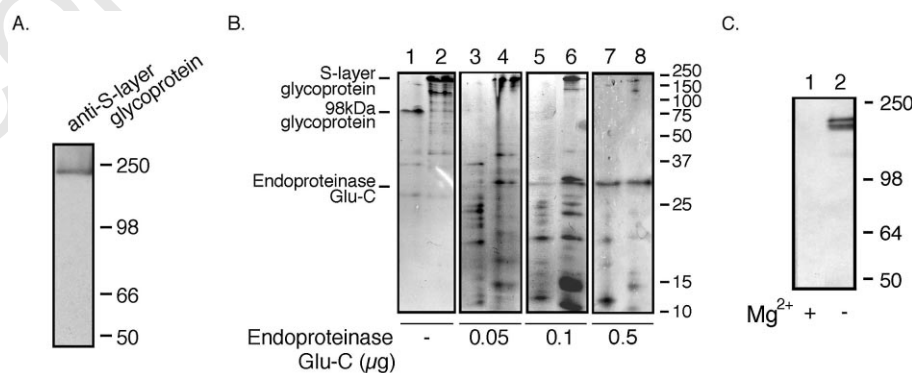


Fig. 2. The *H. volcanii* S-layer glycoprotein and 98-kDa glycoproteins are distinct. A: The protein content of *H. volcanii* cells was probed with antibodies raised against the N-terminal 13 amino acid residues of the S-layer glycoprotein. Molecular mass markers are shown on the right. B: To perform one-dimensional peptide mapping, protein bands containing similar amounts of the 98-kDa glycoprotein (lanes 1, 3, 5 and 7) or S-layer glycoprotein (lanes 2, 4, 6 and 8) were excised from a 7.5% SDS-PAGE gel and added to the wells of a second, 15% gel containing 0 (lanes 1 and 2), 0.05 μg (lanes 3 and 4), 0.1 μg (lanes 5 and 6) or 0.5 μg (lanes 7 and 8) of *Staphylococcus* V8 endoproteinase Glu-C. The positions of the S-layer glycoprotein, the 98-kDa glycoprotein and the endoproteinase are shown on the left, while molecular mass markers are shown on the right. C: To determine whether the 98-kDa glycoprotein is associated with the membrane in a Mg^{2+} -dependent manner, cells were grown in minimal medium containing (lane 1) or lacking Mg^{2+} (lane 2). Growth continued until $\text{OD}_{550} \approx 0.5$, when cells were ^{35}S -radiolabelled for 3 h, and harvested ($8000 \times g$, 30 min). The growth media, corresponding to the uppermost 75% of the supernatant of centrifuged cell cultures, were transferred to fresh tubes and recentrifuged. The uppermost 75% of the second supernatant was concentrated 20-fold in a Centricon-30 spin filter with a molecular mass cutoff of 30 kDa and probed with ConA-Sepharose beads and examined by SDS-PAGE and fluorography. Molecular mass markers are shown on the right.

were once again not coincident (lanes 7 and 8). Thus, the results show that the primary sequences of the S-layer and the 98-kDa glycoproteins differ.

Experiments were next conducted to examine the mode of membrane association of the 98-kDa glycoprotein. The *H. volcanii* S-layer glycoprotein is associated with the membrane in a Mg^{2+} -dependent manner [19,22]. To determine whether Mg^{2+} also plays a role in the membrane attachment of the 98-kDa glycoprotein, cells were grown and radiolabelled in minimal medium containing or lacking Mg^{2+} . Subsequently, both growth media were concentrated, incubated with ConA beads and examined by SDS-PAGE and fluorography (Fig. 2C). Whereas no glycoproteins were released into the growth media of cells grown in the presence of Mg^{2+} (lane 1), cells grown in the absence of magnesium released significant amounts of the 190-kDa S-layer glycoprotein but not the 98-kDa glycoprotein into the growth medium (lane 2). Thus, unlike the S-layer glycoprotein, the 98-kDa glycoprotein is not associated with the membrane in a Mg^{2+} -dependent manner.

3.3. Species-specific differences in the glycosylation of the 98-kDa glycoprotein

To determine whether the 98-kDa glycoprotein is unique to *H. volcanii*, the membrane protein fraction of a closely related strain, *H. mediterranei*, was examined in terms of its glycoprotein content. PAS staining revealed that like *H. volcanii*, *H. mediterranei* also contains a prominent 190-kDa membrane-associated glycoprotein, which most probably corresponds to the S-layer glycoprotein of the latter strain. Both strains also contain similar patterns of more weakly PAS-stained proteins bands. In *H. volcanii*, this list includes the 98-kDa glycoprotein, whereas in *H. mediterranei*, a 95-kDa glycosylated species is present. In contrast to the *H. volcanii* 98-kDa glycoprotein, the *H. mediterranei* 95-kDa glycoprotein was not captured by ConA-Sephrose beads (not shown). The S-layer glycoproteins from both species were, however, captured by the lectin beads. Assuming that the *H. mediterranei* 95-kDa glycoprotein corresponds to the *H. volcanii* 98-kDa glycoprotein, these results reveal that the glycosylation pattern of the protein is modified in a strain-dependent manner.

3.4. Protein glycosylation in the presence of bacitracin and tunicamycin

To gain insight into the mechanism of glycosylation of the 98-kDa glycoprotein, cells were treated with drugs known to interfere with protein glycosylation, i.e. bacitracin and tunicamycin. Bacitracin is a cyclic antibiotic which interferes with the processing of dolichol pyrophosphate, an intermediate involved in the transfer of glycan moieties to protein targets, and has been shown in the case of *Hb.*

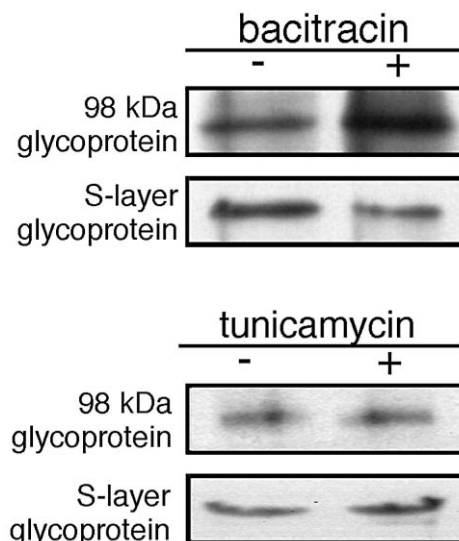


Fig. 3. Neither bacitracin nor tunicamycin affects *H. volcanii* protein glycosylation. Cells were grown in the presence or absence of a sublethal concentration of bacitracin ($25 \mu\text{g ml}^{-1}$) or tunicamycin ($100 \mu\text{g ml}^{-1}$) overnight. The cells were then ^{35}S -radiolabelled ($15 \mu\text{Ci ml}^{-1}$, 90 min) and processed for ConA binding as above.

salinarum to interfere with glycosylation of the S-layer glycoprotein [23]. Tunicamycin interferes with protein glycosylation by interfering with the transfer of UDP-*N*-acetylglucosamine (GlcNAc) to the dolichol phosphate carrier [24] and has been shown to affect protein glycosylation in Archaea [13]. Incubation of *H. volcanii* cells in the presence of the reagents (bacitracin, $25 \mu\text{g ml}^{-1}$; tunicamycin, $50 \mu\text{g ml}^{-1}$) had little effect on protein biosynthesis, as reflected by radiolabel incorporation in the presence or absence of antibiotic (not shown). When the glycoprotein content of pulse-labelled control, bacitracin- or tunicamycin-treated *H. volcanii* cells was captured by ConA-Sephrose beads and subsequently analysed by SDS-PAGE, no effect of the treatments on the apparent molecular masses of the 98-kDa glycoprotein was observed (Fig. 3). Moreover, no effect on the migration of the S-layer glycoprotein was detected either, suggesting the general inability of bacitracin and tunicamycin to affect protein glycosylation in *H. volcanii*, as previously observed [22]. Similarly, bacitracin had no effect on the migration of the S-layer glycoprotein of *H. mediterranei* (not shown).

4. Discussion

Long believed to be a eukaryal trait, it is now clear that Archaea are also capable of protein glycosylation [2,3]. Indeed, eukaryal protein glycosylation is proposed to have originated from the more primitive archaeal system [5]. As such, elucidation of archaeal protein glycosylation could help better describe the more complex eukaryal system. To further understand archaeal protein glycosylation,

the 98-kDa glycoprotein of the halophilic archaeon *H. volcanii* was examined.

Lectin-based chromatography was used for isolation of the 98-kDa glycoprotein. Earlier studies revealed the binding of ConA to *H. volcanii* cells, in general [25], and to the 98-kDa glycoprotein, in particular [14]. While both the extent of glycosylation of the 98-kDa glycoprotein and the structure(s) of the glycan moiety(ies) remain to be determined, it appears that ConA possesses higher affinity for the 98-kDa glycoprotein as compared to the S-layer glycoprotein. Although the S-layer glycoprotein is the major glycoprotein in *H. volcanii* [14,26], ConA treatment led to significant enrichment of the 98-kDa glycoprotein, as reflected by the comparable PAS staining of the lectin-captured S-layer and 98-kDa glycoproteins. The two glycoproteins also differ in amino acid sequence, as suggested by peptide mapping and immunoblotting experiments, as well as in their mode of membrane association. This latter claim is supported by observations that the S-layer glycoprotein but not the 98-kDa glycoprotein are lipid-modified (Z.K. and J.E., in preparation).

Experiments designed at addressing the mechanism of glycosylation of the 98-kDa species were also performed. Bacitracin interferes with the glycosylation of the S-layer glycoprotein of *Hb. salinarum* [23], presumably via interaction of the antibiotic with the dolichyl pyrophosphate carrier used for *N*-glycosylation at the Asn-2 position [1,27], and with glycosylation of flagellins in *Methanococcus deltae* [28]. In contrast, bacitracin does not affect *H. volcanii* S-layer glycoprotein glycosylation [22], nor that of the 98-kDa glycoprotein. The failure of bacitracin to interfere with the protein glycosylation in *H. volcanii* is likely related to the fact that this species apparently contains oligosaccharides linked to dolichol solely via monophosphate bridges [6]; bacitracin does not interact with monophosphate dolichyl species [27]. Tunicamycin, which interferes with the incorporation of GlcNAc early in protein glycosylation, also had no obvious effect on *Haloferax* protein glycosylation. The glycans of the S-layer glycoprotein of *H. volcanii* do not contain GlcNAc [6,29], as apparently do not the oligosaccharides of the 98-kDa glycoprotein. Interestingly, glycosylation of *S. acidocaldarius* glycoproteins was sensitive to tunicamycin treatment [13]. Clearly, Archaea do not rely on a single mechanism of protein glycosylation. Indeed, chemical analysis of the complete glycan structures of several S-layer glycoproteins reveals glycan moieties far more diverse than those of eukaryotes [2,3]. Even between closely related species such as *H. volcanii* and *H. mediterranei*, heterogeneity in glycosylation of the homologous proteins apparently exists, as reflected by differences in ConA binding of their 98- and 95-kDa glycoproteins, respectively.

Further characterization of glycosylated archaeal membrane proteins will assist in understanding of not only protein glycosylation but also of a variety of other archaeal protein processing phenomena, such as the mechanism

of membrane protein targeting and insertion. Presently, very little is understood about how archaeal proteins cross the plasma membrane [30]. The glycoproteins of *H. volcanii* are proposed to undergo glycosylation on the outer surface of the membrane, i.e. after protein translocation. As such, these glycoproteins, including the 98-kDa species addressed in the current report, could serve as reporter proteins in future studies on the archaeal protein translocation process.

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