

Post-translational modification of the S-layer glycoprotein occurs following translocation across the plasma membrane of the haloarchaeon *Haloferax volcanii*

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The halophilic archaeon *Haloferax volcanii* is surrounded by a protein shell solely comprised of the S-layer glycoprotein. While the gene sequence and glycosylation pattern of the protein and indeed the three-dimensional structure of the surface layer formed by the protein have been described, little is known of the biosynthesis of the S-layer glycoprotein. In the following, pulse-chase radiolabeling and cell-fractionation studies were employed to reveal that newly synthesized S-layer glycoprotein undergoes a maturation step following translocation of the protein across the plasma membrane. The processing step, detected as an increase in the apparent molecular mass of the S-layer glycoprotein, is unaffected by inhibition of protein synthesis

and is apparently unrelated to glycosylation of the protein. Maturation requires the presence of magnesium ions, involved in membrane association of the S-layer glycoprotein, and results in increased hydrophobicity of the protein as revealed by enhanced detergent binding. Thus, along with protein glycosylation, additional post-translational modifications apparently occur on the external face of the haloarchaeal plasma membrane, the proposed topological homologue of the luminal face of the eukaryal endoplasmic reticulum membrane.

Keywords: archaea; *Haloferax volcanii*; membrane protein; plasma membrane; post-translational modification.

Archaea, comprising the most recently described yet least well understood domain of life, are best known for their abilities to thrive in drastic environmental conditions such as extremes in temperature, pH and salinity [1–3]. To survive the challenges of their surroundings, archaea have developed various strategies for maintaining protein stability, solubility and activity under conditions that would result in unfolding, aggregation or degradation of proteins from nonextremophilic organisms. While biophysical and structural studies are beginning to offer explanations as to how archaeal proteins are able to properly fold in hyperthermal or hypersaline conditions [4,5], little is known about the biosynthesis of archaeal proteins.

Like their eukaryal counterparts, archaeal proteins are known to undergo a variety of post-translational modifications, including removal of targeting or other precursor-related protein sequences, oligomerization, phosphorylation, attachment of lipid moieties and glycosylation [6–14]. In numerous instances, aspects of archaeal protein modification are reminiscent of the parallel processes in eukarya. In both domains, protein glycosylation involves dolichol-based lipid intermediates [15]. Protein isoprenylation has been reported in archaea and eukarya, but not in eubacteria [16]. In addition, sequence comparisons of signal peptidases, responsible for removing signal sequences from

exported proteins, reveal significant homologies between the archaeal and eukaryal proteins as compared to bacterial versions of the protein, suggesting shared modes of signal cleavage in archaea and eukarya [17]. Topological considerations also reveal similarities between protein processing in archaeal and eukaryal cells. In eukarya, many post-translational protein modification events occur in the lumen of the endoplasmic reticulum (ER). Whereas the subcellular localization of such processes in archaea remains generally unclear, experimental evidence suggests that some of these events, such as protein glycosylation [18–21], take place on the external surface of the archaeal plasma membrane, making this surface the topological homologue of the luminal face of the ER membrane. Thus, addressing archaeal post-translational protein modification will not only describe a poorly understood aspect of archaeal cell biology but could also serve to elucidate parallel modification events in eukarya, given the similarities in mechanism and topology of various post-translational processing events in the two domains.

In the halophilic archaeon *Haloferax volcanii*, the S-layer glycoprotein is the sole component of the proteinaceous shell that surrounds the cell and is responsible for the bowl-like conformation assumed by *H. volcanii* cells [22,23]. The gene encoding the S-layer glycoprotein has been cloned and sequenced and shown to encode for an 828-amino-acid-residue preprotein, including a cleavable 34-amino-acid-residue signal sequence reminiscent of eukaryal and bacterial signal sequences [23]. Furthermore, the chemical composition and attachment sites of the glycan moieties of the protein have also been described [23,24]. Given the different modifications undergone by the protein from the time of translation to its eventual integration into the surface layer that surrounds the cell, the *H. volcanii* S-layer glycoprotein

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Abbreviations: ConA, concanavalin A; ER, endoplasmic reticulum; PAS, Periodic acid-Schiff's reagent.

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is an excellent reporter for following various protein-processing steps in archaea. Accordingly, the following study has addressed the biogenesis of the S-layer glycoprotein. The results of this investigation reveal post-translational modification of the S-layer glycoprotein following translocation of the protein across the plasma membrane. This processing event is apparently unrelated to protein glycosylation, modulates the detergent interaction of the protein and requires the presence of magnesium ions, involved in membrane association of the protein.

EXPERIMENTAL PROCEDURES

Materials

Bacitracin, BSA, DNase, periodic acid, Schiff's reagent, trichloroacetic acid and Triton X-100 were from Sigma (St Louis, MO, USA). Prestained SDS/PAGE molecular mass markers came from Bio-Rad (Hercules, CA, USA). Concanavalin A (ConA)-Sepharose was from Pharmacia (Uppsala, Sweden). Redivue ³⁵S-radiolabeling mixture (> 1000 Ci·mmol⁻¹), D-[U-¹⁴C]-glucose (250 Ci·mmol⁻¹) and D-[U-¹⁴C]-galactose (339 Ci·mmol⁻¹) were from Amersham (Buckingham, UK).

Culture and radiolabeling conditions

H. volcanii DS2 was obtained from the American Type Culture Collection and grown aerobically at 40 °C in minimal medium [25]. For pulse-chase radiolabeling of the cellular protein content, cultures were labeled with 15 μCi ³⁵S per ml for 3 min, after which time 1 mM unlabeled methionine was added. Aliquots were removed immediately prior to and at various intervals following addition of the unlabeled methionine and processed as described in the text. When radiolabeling was performed with either [¹⁴C]glucose or [¹⁴C]galactose, 8–16 μCi of radiolabeled compound were added per ml culture. In pulse-chase experiments, cells were incubated with radiolabeled saccharides for 20 min, after which chase was initiated upon addition of 1 mM unlabeled sugar. The protein content of the radiolabeled cells was precipitated with 15% trichloroacetic acid, pelleted in a microfuge (10 000 g, 15 min, 40 °C), acetone washed, re-pelleted, resuspended in sample buffer [4% SDS, 20% (v/v) glycerol, 0.02% bromophenol blue, 1.5% 2-mercaptoethanol, 125 mM Tris/HCl, pH 6.8] and examined by SDS/PAGE and fluorography using Kodak X-Omat film.

ConA-Sepharose binding

Aliquots of ³⁵S pulse-chase-radiolabeled *H. volcanii* cells were removed at successive intervals during the chase phase and added to 100 μL of 10% (v/v) ConA-Sepharose beads (prewashed in 1 mL lectin buffer: 1 mM Ca²⁺, 1 mM Mn²⁺, 1 mg·mL⁻¹ BSA, 1% Triton X-100, 50 mM Tris/HCl, pH 7.9) and lectin buffer without BSA in a final volume of 1 mL and nutated for 1 h at room temperature. The beads were subsequently washed four times in lectin buffer without BSA and heated (3 min, 95 °C) in the presence of 35 μL sample buffer. The beads were pelleted in a microfuge (5000 g, 3 min), the supernatants were transferred to

clean microtubes and examined by SDS/PAGE and fluorography.

Two-dimensional gel electrophoresis

Radiolabeled proteins were examined by two-dimensional gel electrophoresis by performing nondenaturing gel electrophoresis in the first dimension followed by SDS/PAGE. Nondenaturing gel electrophoresis was performed in the presence of 0.5% Triton X-100 essentially as previously described [26], except that gels were run at 100 V for 7 h. The nondenaturing gel lane was isolated and laid across the top of a 3% stacking gel, overlaid with SDS/PAGE sample buffer, and subjected to 7.5% SDS/PAGE. The resulting gels were then examined by fluorography.

Other methods

Periodic acid-Schiff's reagent (PAS) staining was performed as previously described [27]. Membranes were isolated by osmotic lysis of cells upon transfer into 1 mL water, addition of DNase (6 U) and ultracentrifugation (Beckman TLX Optima tabletop ultracentrifuge; TLA 100.2 rotor, 140 000 g, 15 min, 40 °C). Densitometry was performed using a UMAX Mirage IIse scanner (Willich, Germany) and IPLab Gel software (Signal Analytics, Vienna VI, USA).

RESULTS

Detection of an immature form of the S-layer glycoprotein

To better understand archaeal protein processing, a series of experiments aimed at tracing the biogenesis of the *H. volcanii* S-layer glycoprotein was undertaken. In the first of these, *H. volcanii* cells were subjected to pulse-chase radiolabeling. In these experiments, cells were metabolically ³⁵S-labeled for 3 min after which time an excess of unlabeled methionine was added to the culture. Immediately prior to chase with unlabeled methionine and at various intervals subsequent to the initiation of the chase, aliquots were removed and the behavior of the S-layer glycoprotein was examined by SDS/PAGE and fluorography. Given the absence of cysteine residues in the S-layer glycoprotein [23], addition of unlabeled methionine is sufficient to chase the pulse-labeled S-layer glycoprotein. As shown in Fig. 1A, newly synthesized S-layer glycoprotein first appeared as a single band that was eventually converted into a slower migrating form of the protein. The faster migrating version of the nascent S-layer glycoprotein accounted for the major form of the protein during the first 4 min following the onset of the chase, whereas the slower migrating form of the protein was predominate from the 4-min point onwards (Fig. 1B). The transformation of the faster migrating form of the S-layer glycoprotein into the final, slower migrating version of the protein suggests that these S-layer glycoprotein species correspond to immature and mature versions of the protein, respectively. As the major glycoprotein species in *H. volcanii*, the S-layer glycoprotein can be readily detected by PAS staining [13]. While PAS staining readily revealed the mature form of the S-layer glycoprotein, the immature form

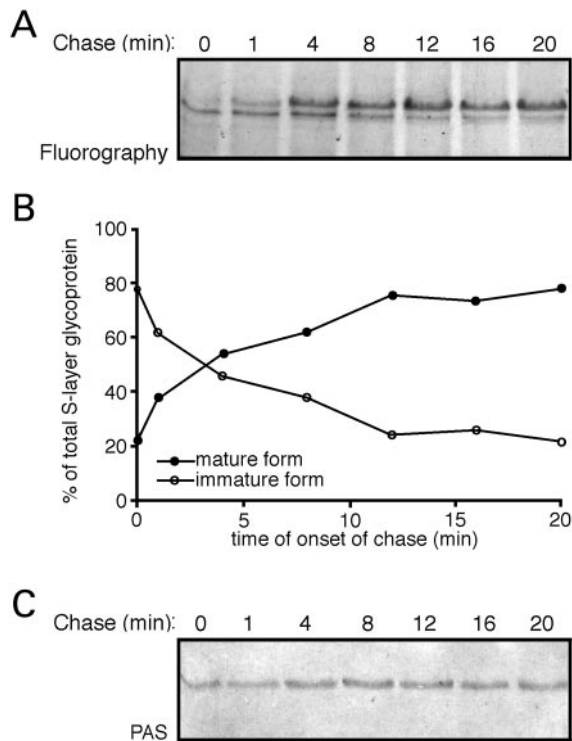


Fig. 1. Maturation of the *H. volcanii* S-layer glycoprotein as revealed by pulse-chase labelling. (A) Cells were ^{35}S metabolically radiolabeled for 3 min followed by addition of an excess of cold methionine. Aliquots were removed immediately prior to addition of cold methionine and then 1, 4, 8, 12, 16, 20, 25 and 30 min following onset of the methionine chase. The samples were then examined by 7.5% SDS/PAGE and fluorography. The period of electrophoresis was optimized to allow optimal separation of the immature and mature forms of the S-layer glycoprotein. (B) Levels of the immature and mature forms of the S-layer glycoprotein were determined densitometrically and expressed as percentages of the combined amount of both versions. (C) Aliquots from a pulse-chase radiolabelling experiment were separated by 7.5% SDS/PAGE and examined by PAS staining.

of the S-layer glycoprotein was not stained, suggesting that either the immature S-layer glycoprotein is not glycosylated or that only minor levels of this version of the glycoprotein are present under steady-state conditions (Fig. 1C).

Maturation of the S-layer glycoprotein occurs following completion of protein translation

The maturation of the nascent S-layer glycoprotein, as revealed by pulse-chase radiolabeling, could reflect elongation of the newly synthesized polypeptide chain upon translation of the most C-terminal portion of the protein. To test the involvement of protein translation in the maturation of the S-layer glycoprotein, anisomycin was added to *H. volcanii* cells during the last minute of a 3-min window of ^{35}S -pulse labeling, followed by chase with an excess of unlabeled methionine. Anisomycin has been reported to inhibit protein translation in haloarchaea [28], and indeed, preliminary studies confirmed that a 1-min pretreatment with the antibiotic inhibited *H. volcanii* protein translation,

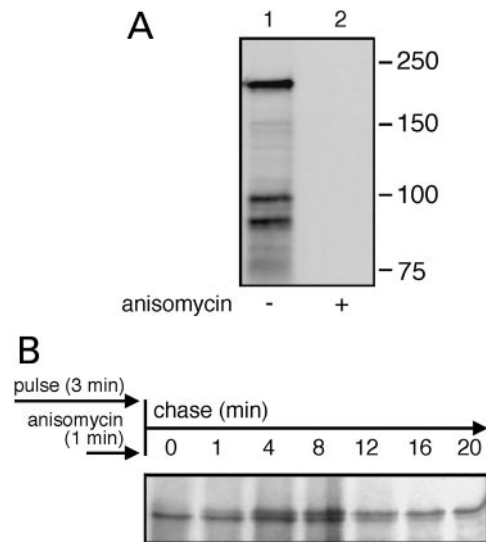


Fig. 2. Maturation of the S-layer glycoprotein occurs following completion of protein translation. (A) *H. volcanii* cells were ^{35}S metabolically radiolabeled ($15\ \mu\text{Ci}\cdot\text{mL}^{-1}$, 3 min) before (lane 1) or after (lane 2) pretreatment with anisomycin ($10\ \mu\text{g}\cdot\text{mL}^{-1}$, 1 min). (B) Anisomycin ($10\ \mu\text{g}\cdot\text{mL}^{-1}$) was added to *H. volcanii* cells during the final minute of a 3-min ^{35}S -radiolabelling pulse, prior to addition of 1 mM unlabeled methionine. Aliquots were removed either immediately before the methionine chase or at various intervals during the chase, processed for SDS/PAGE and examined by fluorography.

as detected by SDS/PAGE and fluorography (Fig. 2A, lane 2). Antibiotic treatment, however, had no effect on the maturation of the S-layer glycoprotein (Fig. 2B). Thus, it can be concluded that protein translation is not involved in maturation of the S-layer glycoprotein.

S-layer glycoprotein maturation occurs on the external surface of the cell

Membrane integration of the S-layer glycoprotein is thought to be mediated through a hydrophobic stretch of 20 amino-acid residues, ending four residues from the C-terminal of the protein (Fig. 3A) [23]. As such, membrane association reflects translocation of the S-layer glycoprotein up to the C-terminal anchoring domain. To determine whether maturation of the S-layer glycoprotein occurred before the protein had traversed the plasma membrane up to the position of the C-terminal membrane anchoring stretch or, rather, occurred prior to translocation of the protein to that region and hence membrane association, subcellular fractionation studies were performed. Membranes were isolated from aliquots taken at various intervals during a pulse-chase experiment and analyzed by SDS/PAGE and fluorography. Even at the earliest time points, the immature form of the S-layer glycoprotein was readily detected in the isolated membrane fraction (Fig. 3B). Examination of membranes isolated from successive aliquots removed from the pulse-chase reaction revealed that the membrane-associated immature S-layer glycoprotein had been eventually converted into the mature form of the protein. Detection of the mature form of the protein in membranes in aliquots isolated from later time points during the chase phase of the experiment confirmed that the

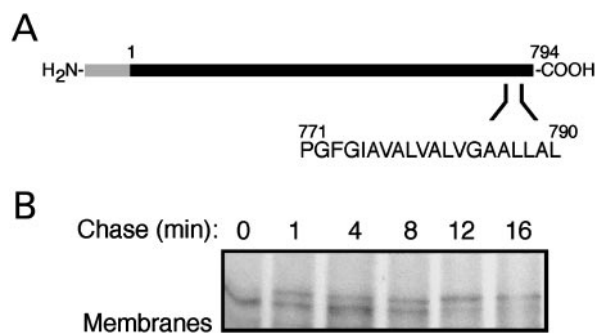


Fig. 3. Maturation of the S-layer glycoprotein occurs following completion of protein translocation. (A) Schematic representation of the S-layer glycoprotein, highlighting the proposed membrane-spanning domain. (B) Aliquots of a pulse-chase experiment were osmotically lysed upon transfer to water, treated with DNase and separated into membrane and soluble fractions by ultracentrifugation. The membrane-containing pellets were resuspended and examined by SDS/PAGE and fluorography.

membrane fraction had indeed been isolated via the experimental approach employed. Indeed, only negligible levels of either form of the nascent S-layer glycoprotein were detected in cytosolic fractions (not shown). Membrane association of the immature version of the S-layer glycoprotein and its eventual conversion to the mature form of the protein in the membrane therefore reflects that protein translocation occurs prior to maturation of the protein.

Both the immature and mature forms of the S-layer glycoprotein are glycosylated

A series of experiments was undertaken to determine whether glycosylation of the immature version of S-layer glycoprotein forms the basis for the increase in apparent molecular mass observed upon maturation. In the first of these studies, the ability of ConA-conjugated Sepharose beads to specifically precipitate the S-layer glycoprotein was employed to determine whether the immature form of the S-layer glycoprotein, like the mature version of the protein [13], was also glycosylated. Aliquots were removed from a pulse-chase radiolabeling experiment at progressive intervals during the chase phase and incubated with ConA-conjugated beads. The lectin-bound material was then examined by SDS/PAGE and fluorography. Although the mature form of the S-layer glycoprotein was seemingly better bound by the ConA-Sepharose beads, the immature form of the protein was also captured by the lectin beads, confirming the glycosylated nature of both versions of the protein (Fig. 4A).

The *H. volcanii* S-layer glycoprotein contains N-linked oligosaccharides, mainly in the form of linear chains of β 1-4-linked glucose residues scattered throughout the length of the mature protein, in addition to O-linked glucosyl-(α 1-2)-galactosyl units [23,24]. Total sugar analysis of the *H. volcanii* S-layer glycoprotein also reveals the presence of smaller levels of mannose and idose [24]. Pulse labeled with either [14 C]glucose or [14 C]galactose resulting in radiolabeling of both the immature and mature forms of the S-layer glycoprotein (Fig. 4B and C). In both cases, the

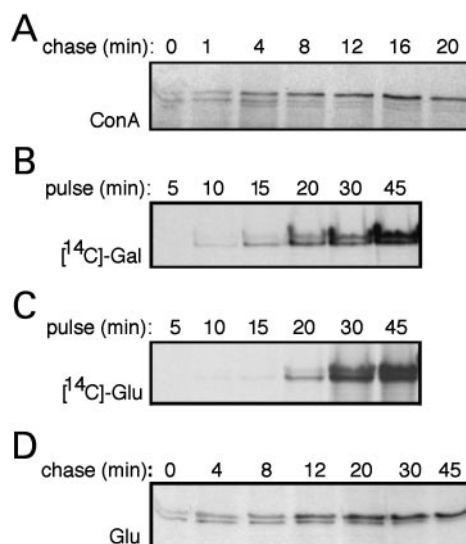


Fig. 4. Both immature and mature versions of the S-layer glycoprotein are glycosylated. (A) Aliquots of a 35 S-pulse-chase radiolabelling experiment were removed at successive intervals and tested for the ability of ConA-Sepharose beads to capture the S-layer glycoprotein. (B) Cells were pulse radiolabeled with [14 C]galactose and aliquots were removed at various intervals, processed for SDS/PAGE and examined by fluorography. (C) As in (B), except that [14 C]glucose was employed. (D) Cells were radiolabeled with [14 C]glucose for 20 min, when 1 mM unlabeled glucose was added. Aliquots were removed at various intervals during the chase period and examined by SDS/PAGE and fluorography. Similar results were obtained with [14 C]galactose.

radiolabel was first detected in the immature version of the protein after approximately 10 min of incubation. Upon continued incubation, radiolabel began to accumulate in the mature form of the S-layer glycoprotein. Pulse-chase radiolabeling confirmed that immature S-layer glycoprotein incorporating the 14 C-radiolabel could be chased into the mature form of the protein (Fig. 4D). The observation that the S-layer glycoprotein appeared as two clearly defined 14 C-radiolabeled bands (and indeed 35 S-radiolabeled bands, as shown above) rather than as a broad and diffuse band spanning the region between the immature and mature versions of the protein argues against the existence of S-layer glycoprotein species containing levels of glycosylation intermediate to those of the immature and mature versions of the protein.

To test further whether protein glycosylation plays a role in the maturation of the S-layer glycoprotein, cells were treated with bacitracin, a membrane-impermeant antibiotic previously shown to interfere with the glycosylation of the S-layer glycoprotein of *H. salinarium* [18,19]. To determine whether bacitracin had any effect on the maturation of the immature form of the S-layer glycoprotein, cells were grown in the absence or presence of the antibiotic ($25 \mu\text{g}\cdot\text{mL}^{-1}$ overnight, or $250 \mu\text{g}\cdot\text{mL}^{-1}$ for 1 h) and radiolabeled in a pulse-chase protocol. No effect on the maturation of the S-layer glycoprotein was observed in the presence of the antibiotic (not shown). Similarly, treatment with tunicamycin, another inhibitor of protein glycosylation, had no effect on the maturation of S-layer glycoprotein (not shown).

Magnesium ions are required for S-layer glycoprotein maturation

The *H. volcanii* S-layer glycoprotein is associated with the membrane in a magnesium-dependent manner, as reflected by selective release of the protein from the cell surface upon incubation with EDTA [23]. Upon removal of magnesium, the bowl-shaped conformation normally assumed by *H. volcanii* cells is lost, presumably due to the shedding of the S-layer glycoprotein. Normal cellular morphology can be restored following restoration of magnesium levels [22]. The role played by magnesium in the biogenesis of the S-layer glycoprotein is, however, unknown. Accordingly, cells were transferred to magnesium-containing or magnesium-free minimal medium, metabolically ^{35}S -radiolabeled and separated into cellular and medium fractions. Examination of the ConA–Sepharose-bound material released into the growth media collected after a 90-min period of radiolabeling by SDS/PAGE and fluorography revealed that only minor levels of radiolabeled S-layer glycoprotein were shed into magnesium-containing medium (Fig. 5A, lane 1) whereas, as expected, substantial release of ^{35}S -incorporat-

ing S-layer glycoprotein into the magnesium-free medium was observed (lane 2). Analysis of the total protein released into magnesium-free medium confirmed the selective release of the S-layer glycoprotein (not shown). The fact that the recently reported 150-, 98-, 58- and 54-kDa *H. volcanii* glycoproteins [13] are not released into the magnesium-free medium suggests that the membrane association of these proteins does not involve magnesium ions. Closer examination of the ConA-bound S-layer glycoprotein captured from magnesium-free medium revealed that the protein existed in two versions, i.e. faster- and slower-migrating forms. Examination of the cell-associated radiolabeled protein content revealed that cells grown in either the presence or absence of magnesium synthesize nascent S-layer glycoprotein as a doublet (lanes 3 and 4, respectively). In the absence of magnesium, however, there was a change in the relative contribution of each component of the doublet to the total amount of S-layer glycoprotein. In magnesium-free medium, the majority of the S-layer glycoprotein (65%, as determined densitometrically) was detected as a faster-migrating version of the protein. In the presence of magnesium, only a very small percentage of the S-layer glycoprotein is detected as a faster-migrating form. The presence or absence of the magnesium had little effect on the electrophoretic behavior of the bulk of other radiolabeled *H. volcanii* proteins.

To relate the effect of magnesium on the composition of the cell-associated S-layer glycoprotein population to the maturation of nascent S-layer glycoprotein, cells were transferred into minimal medium lacking magnesium and subjected to pulse-chase radiolabeling (Fig. 5B). Whereas maturation of the S-layer glycoprotein in cultures grown in control, magnesium-containing medium was essentially completed by 12–16 min (Fig. 5B, top panel), a 2-h depletion of magnesium lead to a prolongation of the S-layer maturation process (Fig. 5B, middle panel). These experiments also show that the faster-migrating version of the S-layer glycoprotein corresponds to the immature version of the protein. In cultures depleted of magnesium for 4 h, only a small percentage of the S-layer glycoprotein matured within the 30-min period of chase (Fig. 5B, lower panel). The comparable degree of incorporation of ^{35}S -containing-amino acids into nascent proteins during the 3-min window of radiolabeling in cultures grown in either the presence or up to 4 h in the absence of magnesium confirmed that the delay in S-layer glycoprotein maturation resulting from the absence of magnesium was not the result of a general decrease in the rate of protein synthesis (not shown). It thus appears that magnesium is required for S-layer glycoprotein maturation.

The mature S-layer glycoprotein is more hydrophobic than the immature version of the protein

To further distinguish between the immature and mature versions of the S-layer glycoprotein, the protein content of pulse-chase radiolabeled cells was examined by two-dimensional gel electrophoresis. Electrophoresis in the first dimension of the radiolabeled protein content of an aliquot removed 4 min following the onset of chase was performed under native conditions in the presence of 0.5% Triton X-100. In the presence of Triton X-100, the migration

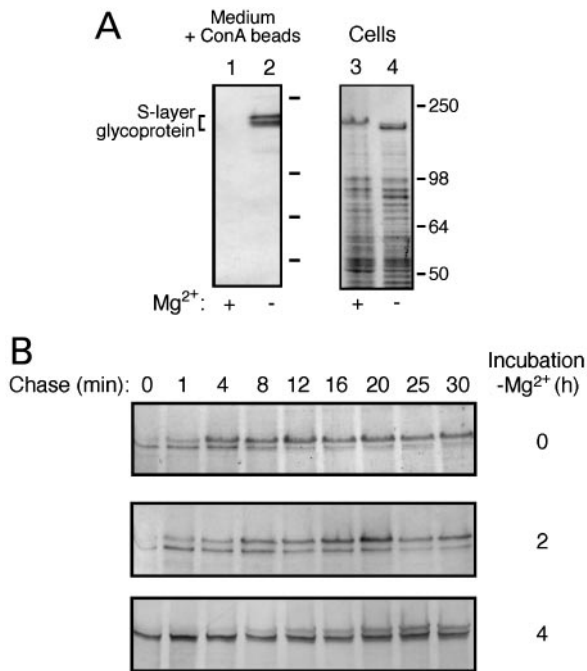


Fig. 5. Magnesium is required for S-layer glycoprotein maturation. (A) Cells were ^{35}S metabolically radiolabeled in minimal medium containing (lanes 1 and 3) or lacking (lanes 2 and 4) magnesium. The radiolabeled cultures were separated into growth media and cellular fractions. The growth media were incubated with ConA–Sepharose beads and examined by SDS/PAGE and fluorography (lanes 1 and 2), whereas cells were directly examined by SDS/PAGE and fluorography (lanes 3 and 4). The position of the S-layer glycoprotein is shown on the left, while the positions of molecular mass markers are shown on the right. (B) Cells were radiolabeled in a pulse-chase experiment following growth in minimal medium containing magnesium (top panel) or following 2 or 4 h of growth in magnesium-free minimal medium (middle and lower panels, respectively). The behavior of the S-layer glycoprotein was then examined by SDS/PAGE and fluorography.

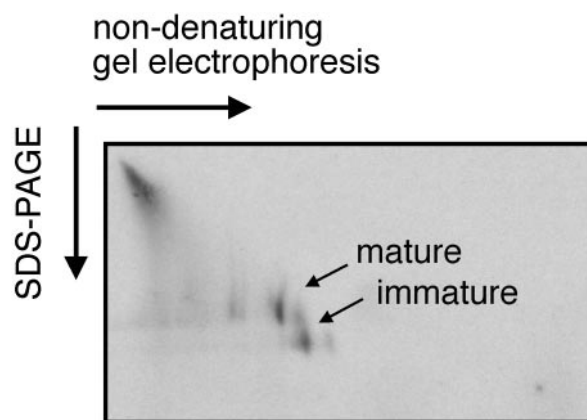


Fig. 6. Two-dimensional gel electrophoresis reveals differences in the hydrophobicity of the mature and immature versions of the S-layer glycoprotein. An aliquot of cells subjected to pulse-chase radiolabelling was removed 4 min after the onset of chase and examined by nondenaturing gel electrophoresis in the presence of 0.5% Triton X-100. The gel lane was cut and applied horizontally to the top of the 3% stacking gel of a 7.5% separating SDS/PAGE gel and electrophoresis was performed as usual. The gel was then dried and examined by fluorography. The directions of electrophoresis under nondenaturing and denaturing (i.e. SDS/PAGE) are shown at the top and to the left of the gel, respectively. Arrows depict the positions of the mature and immature S-layer glycoprotein.

of proteins bearing hydrophobic anchors is retarded by the binding of detergent molecules [26]. To then assess the apparent molecular masses of the proteins separated in the first dimension by nondenaturing gel electrophoresis, SDS/PAGE was performed in the second dimension. Such experiments revealed that the mature form of the S-layer glycoprotein migrated slower in nondenaturing gel electrophoresis than did the immature form of the protein (Fig. 6). The results suggest therefore that the mature version of the S-layer glycoprotein is more hydrophobic than the immature form of the protein, as reflected by the enhanced interaction of the former with detergent molecules. Closer examination reveals that the immature version of the S-layer contains a minor population that migrates slightly faster than the bulk of the immature form in the first direction of electrophoresis, i.e. nondenaturing gel electrophoresis. Thus, the immature form of the protein may undergo an additional processing step prior to maturation.

DISCUSSION

The S-layer glycoprotein is the sole component of the proteinaceous shell surrounding *H. volcanii* cells [23]. During the first minutes of its biogenesis, nascent S-layer glycoprotein undergoes a post-translational processing event whereby an immature version of the S-layer glycoprotein is converted into a mature form of the protein possessing higher apparent molecular mass. Modification of the immature S-layer glycoprotein is a plasma membrane-associated process as no S-layer glycoprotein maturation was detected in cells grown in the absence of magnesium, a condition in which the S-layer glycoprotein is selectively released into the growth medium. The concept that post-translational modification of the S-layer glycoprotein is

localized to the membrane is supported by the finding that maturation occurs following translocation of the protein up to the C terminal membrane-anchoring domain of the protein.

Protein glycosylation was considered as the basis for the S-layer glycoprotein maturation event, given that the glycan moieties of the *H. volcanii* S-layer glycoprotein have been calculated to add 12% to the predicted molecular mass of the protein [23] and that experimental evidence suggests that glycosylation of nascent haloarchaeal proteins occurs on the external surface of the plasma membrane, during or after translocation [18–21]. Several observations suggest, however, that protein glycosylation is not responsible for S-layer glycoprotein maturation. Lectin-binding studies confirmed that both the immature and mature forms of the S-layer glycoprotein are glycosylated. Thus, the inability of PAS staining to detect the glycosylated immature form of the S-layer glycoprotein suggests that this version of the protein only represents a minor population. The glycosylated nature of both versions of the S-layer glycoprotein was also shown upon incubation of cells with either [^{14}C]glucose or [^{14}C]galactose, which resulted in radiolabeling of both versions of the S-layer glycoprotein. The possibility that the radiolabel had been transferred to noncarbohydrate structures and only then incorporated into the S-layer glycoprotein cannot, however, be discounted as it is not yet possible to isolate sufficient quantities of the immature version of the S-layer glycoprotein for direct testing of the incorporation of radiolabeled sugar. While both versions of the protein are glycosylated, it is unlikely that additional glycosylation of the immature form of the protein is responsible for the maturation event, given the failure to detect forms of the S-layer glycoprotein containing levels of glycosylation intermediate to those of the immature and mature versions of the protein. Finally, lectin binding of the S-layer glycoprotein released into magnesium-free medium, i.e. conditions in which maturation is impaired, suggests that glycosylation and maturation are unrelated. Interestingly, bacitracin, shown to modulate protein glycosylation and hence SDS/PAGE migration of the mature S-layer glycoprotein from *H. salinarium* [18,19], had no effect on either the glycosylation or maturation of the *H. volcanii* S-layer glycoprotein. This could be related to the finding that while *H. volcanii* relies on dolichol-based intermediates for oligosaccharide transfer, only dolichol intermediates containing monophosphate-linked oligosaccharides are detected in this species [29]; bacitracin selectively interferes with the processing of pyrophosphate-containing dolichol species [30]. Similarly, tunicamycin had no effect on S-layer glycoprotein maturation. Indeed, the effects of bacitracin and tunicamycin on archaeal protein glycoprotein biosynthesis are not universal. Glycosylation of flagellar glycoproteins of the methanoarchaeon *Methanococcus deltae* was unaffected by either bacitracin or tunicamycin treatment [31], whereas glycosylation of the *Sulfolobus acidocaldarius* S-layer glycoprotein was inhibited by the latter [32].

Two-dimensional gel electrophoresis revealed that the two versions of the S-layer glycoprotein can be distinguished in terms of hydrophobicity, with the mature form of the protein being more hydrophobic than the immature form. Interestingly, it was recently reported that the S-layer glycoprotein of *H. salinarium* (*halobium*) is anchored to the plasma membrane through a diphitynylglycerolphosphate

lipid anchor, in addition to the previously described membrane-spanning stretch of the protein [12]. While the precise site of the isoprenylation of the *H. salinarium* protein remains unknown, the modification has been localized to the C-terminal region of the protein, proximal to the transmembrane domain. In many aspects, the C-terminal region of the *H. volcanii* S-layer glycoprotein resembles its *H. salinarium* counterpart. For instance, both contain a cluster of O-glycosylated threonine residues just upstream of very similar putative membrane-spanning stretches. Thus, lipid modification offers a plausible explanation for the maturation of the *H. volcanii* S-layer glycoprotein. Accordingly, the presence of covalently bound lipid moieties has been previously shown to retard protein migration in nondenaturing gel electrophoresis [26], as observed in the present study in the case of the mature version of the *H. volcanii* S-layer glycoprotein.

In addition to maturation, the S-layer glycoprotein apparently undergoes additional processing events. Although *H. volcanii* possess a generation time of 2.5–3 h under optimal conditions [33], biosynthesis of the S-layer glycoprotein occurs within minutes, suggesting that significant turnover of the protein occurs at the cell surface. Indeed, it has been observed that despite the fact that the mature form of the nascent S-glycoprotein migrates coincidentally with the existing pool of the protein in SDS/PAGE (Fig. 1C), differences in the protease sensitivities of the two populations exist, suggesting conformational differences [13]. Such conformational changes may be necessary for incorporation of nascent S-layer glycoprotein into the existing S-layer structure. It is conceivable that magnesium ions, required for anchoring of the immature S-layer glycoprotein and hence for S-layer glycoprotein maturation, could also be involved in the incorporation process through direct modulation of membrane anchoring of the protein or by affecting another aspect of S-layer architecture.

Topologically, S-layer glycoprotein maturation is reminiscent of eukaryal post-translational processing events. In eukarya, numerous protein modifications, including protein glycosylation, lipid modification and oligomerization, take place following translocation of the protein across the membrane of the ER. Once inside the ER lumen, proteins experience surroundings that are distinct from the cytoplasmic environment and conducive to a variety of protein-processing events [34]. Electron microscopy modelling of *H. volcanii* cell-surface architecture suggests the presence of a pseudo-periplasmic space between the surface layer, comprised solely of the S-layer glycoprotein, and the plasma membrane [35]. The existence of such a compartment could create a protected microenvironment in which various post-translational modifications, including those involved in the maturation of the S-layer glycoprotein, could occur.

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