

Mini-Review

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Facing extremes: archaeal surface-layer (glyco)proteins

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Archaea are best known in their capacities as extremophiles, i.e. micro-organisms able to thrive in some of the most drastic environments on Earth. The protein-based surface layer that envelopes many archaeal strains must thus correctly assemble and maintain its structural integrity in the face of the physical challenges associated with, for instance, life in high salinity, at elevated temperatures or in acidic surroundings. Study of archaeal surface-layer (glyco)proteins has thus offered insight into the strategies employed by these proteins to survive direct contact with extreme environments, yet has also served to elucidate other aspects of archaeal protein biosynthesis, including glycosylation, lipid modification and protein export. In this mini-review, recent advances in the study of archaeal surface-layer (glyco)proteins are discussed.

Overview

The fascinating ability of members of the *Archaea* to thrive in extremes of temperature, salt and pH as well as in other seemingly hostile niches has generated substantial interest in the molecular mechanisms responsible for mediating survival in the face of such environmental challenges. One aspect of such investigation asks how the archaeal cell envelope, directly exposed to the harsh physical conditions in which these micro-organisms exist, manages to maintain its structural integrity. In most cases, a surface (S)-layer, generally formed from identical protein subunits arranged into a monolayer of simple and repetitive patterns, serves as the envelope of the archaeal cell. Research into the biogenesis of S-layer (glyco)proteins has, however, not only provided insight into the molecular tactics adopted by these polypeptides for survival in the drastic surroundings in which they live, but has advanced understanding of various aspects of archaeal protein biology. For instance, glycosylated S-layer proteins, the major, if not sole, component of many archaeal S-layers, presently represent amongst the best-characterized of all prokaryotic glycoproteins and have served as useful reporters of the archaeal glycosylation process. In addressing the biosynthesis of archaeal S-layer (glyco)proteins, the existence of other protein modifications previously thought to be restricted to eukarya, such as isoprenylation, has been revealed in archaea. Finally, the synthesis of S-layer (glyco)proteins represents an excellent model system in efforts aimed at elucidating the mechanism of protein export in archaea. In the following, recent advances in deciphering both the molecular adaptations of archaeal S-layer (glyco)proteins to extreme conditions as well as the biogenesis of archaeal S-layer (glyco)proteins are presented.

Molecular adaptations of archaeal S-layer (glyco)proteins to life in extreme environments Thermoarchaeal S-layer (glyco)proteins

The S-layers of thermophilic and hyperthermophilic archaea must maintain their integrity and structural attributes in the face of elevated temperatures. Recent comparison of S-layer (glyco)proteins in a single genus containing members found across a broad range of temperatures has allowed for an examination of rules governing the heat-stability of S-layer (glyco)proteins in thermoarchaea. Analysis of S-layer protein sequences from the mesophilic methanarchaea *Methanococcus voltae* and *Methanococcus vannielii* (optimal growth at 37 °C), from the thermophile *Methanothermococcus (Methanococcus) thermolithotrophicus* (optimal growth at 65 °C) and from the hyperthermophile *Methanocaldococcus (Methanococcus) jannaschii* (optimal growth at 85 °C) revealed only minor differences in S-layer protein composition at the amino acid and secondary structural levels (Akça *et al.*, 2002; Claus *et al.*, 2002). However, some trends were observed. While archaeal S-layer (glyco)proteins are generally characterized by a predominance of non-polar residues, examination of overall amino acid composition revealed that the thermophilic and hyperthermophilic species contain an enhanced number of charged residues (Akça *et al.*, 2002; Claus *et al.*, 2002). A similar tendency is also noted in S-layer protein amino acid sequences in other thermophilic and hyperthermophilic species (Akça *et al.*, 2002; Claus *et al.*, 2002). The S-layer glycoproteins of the hyperthermophiles *Methanothermus fervidus* and *Methanothermus sociabilis* appear, however, to be exceptions to the rule, containing more asparagine than aspartate residues (Bröckl *et al.*, 1991). Further charges could be introduced upon glycosylation of thermoarchaeal

S-layer proteins. While sequence analysis suggests *N*-glycosylation of thermophilic archaeal S-layer proteins, direct evidence of oligosaccharide modification has only been shown in a limited number of instances, i.e. in the case of *Methanothermobacter fervidus* (Kärcher *et al.*, 1993), *Methanothermobacter sociabilis* (Bröckl *et al.*, 1991), *Sulfolobus* spp. (Grogan, 1989) and *Staphylothermus marinus* (Peters *et al.*, 1995) S-layer proteins. It has been proposed that an increase in charged residues could attribute thermal stability to thermophilic S-layer (glyco)proteins through coulombic interactions of enhanced stability that occur at higher temperatures or due to enhanced electrostatic interactions (cf. Claus *et al.*, 2002). Indeed, general comparisons of thermophilic and mesophilic proteins reveal an equal increase in oppositely charged residues in the thermophilic sequences (Cambilliau & Claverie, 2000; Das & Gerstein, 2000). At the structural level, the S-layer (glyco)proteins of hyperthermophilic methanoarchaeal strains are predicted to contain a higher proportion of β -structures than their mesophilic counterparts, a modification also believed to impart thermal stability (Bröckl *et al.*, 1991; Akça *et al.*, 2002). Finally, whereas the cysteine content of S-layer (glyco)proteins is generally low (Sára & Sleytr, 2000), *Methanothermobacter fervidus*, *Methanothermobacter sociabilis* and *Methanocaldococcus jannaschii* S-layer proteins contain multiple cysteine residues, possibly hinting at disulfide bridges contributing to thermal stability of their S-layer (glyco)proteins or the S-layer formed therefrom.

Haloarchaeal S-layer glycoproteins

While S-layer (glyco)proteins have been identified in archaea living in a wide range of physical conditions, the best characterized are the S-layer glycoproteins of halophilic archaea. To date, three haloarchaeal S-layer glycoproteins have been studied in detail, i.e. the S-layer glycoproteins of *Halobacterium salinarum* (Lechner & Sumper, 1987), *Haloferax volcanii* (Sumper *et al.*, 1990) and *Haloarcula japonica* (Wakai *et al.*, 1997). As with many haloarchaeal proteins, haloarchaeal S-layer glycoproteins are enriched in acidic residues relative to their non-halophilic counterparts. An enhanced number of acidic residues is thought to encourage proper protein folding in high-salt conditions (Madern *et al.*, 2000; Fukuchi *et al.*, 2003). In each case, the S-layer glycoproteins are believed to be anchored to the plasma membrane via a single-transmembrane domain located near the C-terminus. The three haloarchaeal S-layer glycoproteins share significant sequence similarity throughout their lengths, except as one approaches the N-terminus. Such sequence divergence does not, however, seem to translate into structural differences, given the inability of computer-aided reconstructions to detect any significant dissimilarities between the *Halobacterium salinarum* and *Haloferax volcanii* S-layers (Trachtenberg *et al.*, 2000). In terms of glycosylation, haloarchaeal S-layer glycoproteins undergo both *N*- and *O*-glycosylation (Lechner & Sumper, 1987; Sumper *et al.*, 1990). In the *Halobacterium salinarum* and *Haloferax*

volcanii S-layer glycoproteins, *N*-glycosylation sites are distributed throughout the length of the sequence, whereas *O*-glycosylation sites are clustered in a threonine-rich region immediately preceding the C-terminal membrane-spanning domain. Despite overall similarities at the amino acid level, striking differences in *N*-glycosylation are, however, observed across strain lines, and are thought to reflect differences in the salinity encountered by each strain (Mengele & Sumper, 1992). Whereas only neutral oligosaccharides are present in the S-layer glycoprotein of *Haloferax volcanii*, considered a moderate halophile (optimal growth at 1.7–2.5 M NaCl), the S-layer glycoprotein in the extreme halophile *Halobacterium salinarum* (optimal growth at 3–4 M NaCl) contains sulfated glucuronic acid residues and a higher degree of glycosylation. It is thought that these differences, leading to an increased density in surface charges in the latter strain, reflect an adaptation in response to the higher salt concentrations experienced by *Halobacterium salinarum*. In the *Haloarcula japonica* S-layer glycoprotein, five *N*-glycosylation sites, clustered towards the C-terminus, are predicted, although the character of the putative oligosaccharide has yet to be described (Wakai *et al.*, 1997).

Biogenesis of archaeal S-layer (glyco)proteins

Export

Archaeal S-layer (glyco)proteins are synthesized with an N-terminal extension not found in the mature protein. Closer examination of these sequences reveals similarities to type I signal peptides recognized by the Sec protein translocation system and responsible for the transfer of proteins across the bacterial plasma membrane and the eukaryal endoplasmic reticulum (ER) membrane (Nielsen *et al.*, 1999; Bardy *et al.*, 2003). In *Halobacterium salinarum*, the S-layer glycoprotein is synthesized in a precursor form including a 34-residue signal peptide (Lechner & Sumper, 1987), while signal peptides of similar length and composition are also found in the precursors of the S-layer glycoproteins of *Haloferax volcanii* (Sumper *et al.*, 1990) and *Haloarcula japonica* (Wakai *et al.*, 1997). In *Methanocaldococcus voltae*, it was originally proposed that the S-layer protein was synthesized with a signal peptide of only 12 amino acid residues, lacking traits common to type I signal peptides (Dharmavaram *et al.*, 1991; Konisky *et al.*, 1994). This concept has been recently challenged, however, based on alignments of a larger sample of methanococci S-layer (glyco)protein sequences (Akça *et al.*, 2002). In most instances, archaeal S-layer (glyco)protein signal peptide cleavage sites have been predicted using computer programs trained on bacterial or eukaryal exported proteins (Nielsen *et al.*, 1999; Bardy *et al.*, 2003). In only a limited number of cases have the predicted signal peptide cleavage sites been confirmed experimentally (Dharmavaram *et al.*, 1991; Akça *et al.*, 2002; Irihimovitch & Eichler, 2003). Indeed, cleavage of the S-layer glycoprotein signal peptide forms the basis of *in vitro* assays addressing the function of archaeal type I signal peptidase, the enzyme responsible for

removal of the signal peptide following translocation (V. Irihimovitch, Z. Konrad & J. Eichler, unpublished; Ng & Jarrell, 2003). Finally, as with other exported archaeal proteins, the temporal relationship between the translation and translocation of archaeal S-layer (glyco)proteins remains to be clarified. Recently, *Haloferax volcanii* cells transformed to express fusion proteins formed from this species' S-layer glycoprotein signal peptide fused to different reporter proteins revealed a post-translational mode of translocation (Irihimovitch & Eichler, 2003). It, however, remains to be determined whether this relation also holds true for the native S-layer glycoprotein in this and other archaeal strains.

Glycosylation

The S-layer glycoprotein of *Halobacterium salinarum* was the first non-eukaryal glycoprotein to be described in detail (Lechner & Sumper, 1987). Since, the glycan profile of additional archaeal S-layer glycoproteins has been described. Detailed descriptions of the molecular composition of these moieties, their modes of attachment to S-layer proteins as well as other questions related to archaeal saccharide chemistry have been treated in a series of recent reviews (Sumper & Wieland, 1995; Messner, 1997; Moens & Vanderleyden, 1997; Schäffer & Messner, 2001; Messner & Schäffer, 2003). Here, general lessons on archaeal protein glycosylation learned through analysis of S-layer protein glycosylation are considered.

In addressing archaeal S-layer glycoprotein biogenesis, species-specific aspects of the archaeal glycosylation process have been revealed. In *Sulfolobus acidocaldarius*, tunicamycin, known to interfere with the transfer of UDP-*N*-acetylglucosamine to dolichol phosphate polysaccharide carriers (Elbein, 1981), hinders S-layer glycoprotein glycosylation (Grogan, 1996). In contrast, tunicamycin has no effect on the biosynthesis of the *Haloferax volcanii* S-layer glycoprotein (Eichler, 2001). Similarly, bacitracin, shown to interfere with glycosylation of the *Halobacterium salinarum* S-layer glycoprotein (Mescher & Strominger, 1976), also had no effect on *Haloferax volcanii* S-layer glycoprotein biosynthesis (Eichler, 2001). In *Halobacterium salinarum*, bacitracin is thought to interfere with the processing of the dolichyl pyrophosphate carrier used for glycosylation at the Asn-2 position of the S-layer glycoprotein (Wieland *et al.*, 1981). The failure of the antibiotic to modify *Haloferax volcanii* S-layer glycoprotein biogenesis is likely related to the fact that, unlike in *Halobacterium salinarum*, where both monophosphate- and pyrophosphate-linked dolichol oligosaccharide carriers are present (Lechner & Wieland, 1989), only monophosphate-linked oligosaccharide-dolichol intermediates are detected in *Haloferax volcanii* (Kuntz *et al.*, 1997).

Exploring S-layer glycoprotein glycosylation has also revealed the sophistication of the archaeal protein glycosylation machinery. The *Halobacterium salinarum* S-layer glycoprotein contains two different types of *N*-linked oligosaccharide

chains, with sulfated glucuronic acid moieties attached to asparagine-linked glucose residues predominating and a single chain of a sulfated repeating unit pentasaccharide linked through *N*-acetylgalactosamine positioned at the 2-asparagine position of the protein (Lechner & Wieland, 1989). It remains unclear how the archaeal glycosylation machinery determines which oligosaccharide entity is to be attached to a particular glycosylation site. Moreover, it could be shown that replacement of the serine residue at position 4 of the protein by a valine, leucine or asparagine residue did not interfere with normal S-layer glycoprotein glycosylation, suggesting that motifs apart from the consensus Asn-Xaa-Ser/Thr sequence are recognized by the haloarchaeal glycosylation machinery (Zeitler *et al.*, 1998).

Finally, several studies addressing S-layer glycoprotein glycosylation suggest that in archaea, protein glycosylation occurs on the outer cell surface. Despite being unable to permeate the plasma membrane, bacitracin selectively prevented *Halobacterium salinarum* S-layer glycoprotein glycosylation (Mescher & Strominger, 1976). The ability of *Halobacterium salinarum* cells to add sulfated oligosaccharides, such as those that decorate the S-layer glycoprotein in this species, to exogenously added cell impermeant hexapeptides containing an *N*-glycosylation motif further supports the assignment of the haloarchaeal glycosylation machinery to the external cell surface (Lechner & Wieland, 1989). Similarly, in studies relying on the glucosyltransferase inhibitors amphomycin, PP36 and PP55, it was concluded that glycosylation of *Haloferax volcanii* glycoproteins, including the S-layer glycoprotein, occurs on the outer cell surface (Zhu *et al.*, 1995). Hence it appears that glycosylation of haloarchaeal S-layer glycoproteins is topologically homologous to the eukaryal protein glycosylation process, as in both cases translocation across a membrane precedes protein modification. In eukarya, protein glycosylation only begins once a protein has traversed the membrane of the ER (Kornfeld & Kornfeld, 1985).

Lipid modification

In addition to glycosylation, archaeal S-layer (glyco)proteins may also experience additional post-translational modifications. In studies addressing the biosynthesis of the *Haloferax volcanii* S-layer glycoprotein, it was reported that the protein is first synthesized as an immature precursor, possessing a lower apparent molecular mass and less hydrophobic character than the final version of the protein (Eichler, 2001). The post-translational conversion to the mature form apparently involves isoprenylation, since the S-layer glycoprotein can be labelled with [³H]mevalonic acid, an isoprene precursor (Konrad & Eichler, 2002). This observation is in line with earlier findings that the *Halobacterium salinarum* S-layer glycoprotein is modified by a covalently linked diphytanylglycerol phosphate moiety (Kikuchi *et al.*, 1999). Indeed, the *Halobacterium salinarum* S-layer glycoprotein also undergoes maturation similar to that experienced by its *Haloferax volcanii* counterpart, suggesting that

isoprenylation may be a general feature of haloarchaeal S-layer glycoproteins (Konrad & Eichler, 2002). A combination of pulse-chase radiolabelling and subcellular fractionation approaches has shown that lipid modification of the *Haloferax volcanii* S-layer glycoprotein only occurs once the S-layer glycoprotein has been delivered across the plasma membrane (Eichler, 2001). Like protein glycosylation, lipid modification of eukaryal proteins also occurs at the luminal face of the ER membrane, i.e. once the protein has translocated across the membrane (Wang *et al.*, 1999). This, therefore, reveals further functional parallels between the archaeal external surface and the luminal face of the ER membrane. Still, it is not clear why haloarchaeal S-layer glycoproteins would require an isoprene-based link to the membrane in addition to the C-terminal membrane-spanning domain. One possibility would be to offer physical support to the S-layer itself, thereby creating a periplasm-like space (Kessel *et al.*, 1988; Peters *et al.*, 1995). Alternatively, lipid modification of membrane-inserted haloarchaeal S-layer glycoproteins could reflect a primitive version of a mode of protein membrane anchoring prevalent in eukarya.

Turnover

In at least one archaeal species, the S-layer includes additional proteins that may be involved in the breakdown of S-layer (glyco)proteins. In *Staphylothermus marinus*, the S-layer is composed of an S-layer glycoprotein together with a lighter, 85 kDa chain, both originating from the same gene (Peters *et al.*, 1995). In addition, a 130 kDa globular glycoprotein, shown to be a subtilisin-like protease functional at elevated temperatures in the presence of detergent or denaturants, is also present (Mayr *et al.*, 1996). Although capable of digesting the S-layer glycoprotein, the physiological function of the protease is not clear. It is tempting to speculate that remodelling of the archaeal S-layer via degradation of S-layer proteins is a normal part of various cellular functions involving the S-layer, such as cell division (Pum *et al.*, 1991), morphological changes (Mayerhofer *et al.*, 1998) or transfer of genetic material (Rosenshine *et al.*, 1989; Schleper *et al.*, 1995).

Conclusions

As an increasing number of archaeal genome sequences become available, attention has begun to focus on the archaeal proteome. Such post-genomic studies are beginning to uncover the rules governing the ability of archaeal proteins to properly fold and function in the face of conditions which would normally lead to protein denaturation. Moreover, such studies are helping to reveal the various modifications experienced by archaeal proteins during their lifetimes. As discussed in this mini-review, examination of archaeal S-layer proteins is at the forefront of such efforts. Continued investigation will, however, not only advance our understanding of archaeal protein biology, but will also encourage the use of archaeal S-layers in a multitude of

applications requiring a monolayer network of defined composition, able to remain stable in a variety of physical conditions.

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