Post-translational secretion of fusion proteins in the halophilic archaeon

*Haloferax volcanii*

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running title: Post-translational secretion in *H. volcanii*
ABSTRACT

Whereas protein secretion occurs post-translationally in Bacteria and is mainly a co-translational event in Eukarya, the relationship between the translation and translocation of secreted proteins in Archaea is not known. To address this question, the signal peptide-encoding region of the surface layer glycoprotein gene from the haloarchaeon *Haloferax volcanii* was fused to either the cellulose-binding domain of the *Clostridium thermocellum* cellulosome or to the cytoplasmic enzyme dihydrofolate reductase from *Haloferax volcanii*. Signal peptide-cleaved, mature versions of both the cellulose-binding domain and dihydrofolate reductase could be detected in the growth medium of transformed *Haloferax volcanii* cells. Immunoblot analysis, however, revealed the presence of full-length, signal peptide-bearing forms of both proteins inside the cytoplasm of the transformed cells. Proteinase accessibility assays confirmed that the presence of cell-associated, signal peptide-bearing proteins was not due to medium contamination. Moreover, the pulse-radiolabeled signal peptide-cellulose-binding domain chimera could be chased from the cytoplasm into the growth medium even following treatment with anisomycin, an antibiotic inhibitor of haloarchaeal protein translation. Thus, these results provide evidence that in Archaea, at least some secreted proteins are first synthesized inside the cell and only then translocated across the plasma membrane into the medium.
INTRODUCTION

Correct protein localization is essential for the proper working of the cell. In the case of proteins destined to reside beyond the cytoplasm, such localization requires transfer across lipid-based membranes. In both Bacteria and Eukarya, secreted proteins are synthesized as precursors, or ‘preproteins’, containing a cleavable N-terminal extension referred to as the signal peptide (SP)\(^1\) (1). The signal peptide, not found in the mature polypeptide, serves to target the preprotein to membrane-embedded translocation complexes (2). While the signal peptides of bacterial and eukaryal secretory proteins, incorporating a positively-charged region followed by a hydrophobic core and then a polar cleavage region, are often highly similar and even interchangeable (3), the temporal relationship between secretory protein translation and translocation differ between these two domains of life. In Bacteria, secreted proteins are translocated post-translationally, i.e. once most, if not all, of the protein has been translated in the cytoplasm (4,5). In contrast, the translocation of secretory proteins across the membrane of the eukaryal endoplasmic reticulum, the topological homologue of the bacterial plasma membrane, is coupled to protein translation (1), although post-translational translocational has been reported in Eukarya (6-8).

Whereas the process of protein translocation is well-described in Bacteria and Eukarya (9-12), little is known about how proteins cross the plasma membrane of Archaea, the third and most recently described domain of life. Archaea encode for components of the translocation complexes found in the bacterial and eukaryal systems and exported archaeal proteins often contain signal peptides similar to those found in the other two domains (13,14). Numerous other aspects of the archaeal secretion process have, however, yet to be addressed. In particular, the interplay between translation and translocation during archaeal protein export remains to be
elucidated. Archaea contain a signal recognition particle (SRP), the agent responsible for linking translation and translocation in Eukarya (15), reminiscent of its eukaryal counterpart (16). Many Archaea, however, also encode for homologues of SecDF (17), proteins which in Bacteria serve to modulate the membrane association of SecA, the translocation complex component responsible for coupling ATP energy to the post-translational movement of preproteins across the plasma membrane (18,19). Searches of completed archaeal genomes, however, have thus far failed to reveal an archaeal SecA homologue. Thus, questions concerning the driving force of archaeal translocation also remain unanswered.

Studies addressing the relationship between archaeal protein translation and translocation to date have mainly focused on the biosynthesis of the *Halobacterium salinarum* multi-spanning membrane protein, bacterioopsin. Based on the co-sedimentation of 7S RNA and bacterioopsin mRNA with membrane-bound polysomes, as well as more recent *in vivo* kinetic analysis, a co-translational, SRP-dependent mode of protein translocation is suggested in Archaea (20,21). In contrast, studies following the membrane insertion of a chimeric version of bacterioopsin expressed in *Haloferax volcanii* reported that the fusion protein is first synthesized in the cytoplasm and only then inserted into the membrane (22). Studies relying on newly-synthesized bacterioopsin as a reporter of the relation between translation and translocation in Archaea must be, however, viewed with caution, given this protein’s unusually short, 13 residue signal peptide that lacks a hydrophobic core and contains negatively-charged glutamate residues, rather than the positively-charged amino acids found in most signal peptides (20), as well as the possibility of a dedicated system for bacterioopsin membrane insertion (22). Moreover, the interaction between translation and translocation of a membrane protein may differ in the case of protein secretion.
Thus, to elucidate the temporal relation between translation and translocation during archaeal protein secretion, this work addressed the heterologous expression of two chimeric preproteins composed of the SP of the surface layer glycoprotein (SLG) of *H. volcanii* fused to either the cellulose binding-domain (CBD) of the *Clostridium thermocellum* cellulosome or to the cytoplasmic *H. volcanii* enzyme dihydrofolate reductase-1 (DHFR-1). The molecular composition of the 34 amino acid residue-long SP of the SLG is similar to secretory preproteins in other domains of life (2,3) and, as previously observed, includes a cleavage site recognized by type I signal peptidases (25). Our studies reveal that in *H. volcanii*, these chimeric preproteins are first completely synthesized within the confines of the cell and are only then translocated across the plasma membrane into the medium, with the SP being most likely cleaved by the externally-oriented, membrane-bound signal peptidase.
MATERIALS AND METHODS

Materials

Ampicillin, anisomycin, cellulose, DNase I, novobiocin, PEG-600 and Triton X-100 came from Sigma (St. Louis, MO). Proteinase K came from Boehringer (Mannheim, Germany). Yeast extract came from Pronadisa (Madrid, Spain) while tryptone came from USB (Cleveland, OH). Molecular weight markers and goat anti-rabbit horseradish peroxidase-conjugated antibodies were from BioRad (Hercules, CA). Redivue \[^{35}S\] radiolabelling mixture (>1000 Ci/mmol) and an ECL kit came from Amersham (Buckingham, UK).

Organisms and growth conditions

The *H. volcanii* methionine/cysteine auxotrophic strain WR341 (22), as well as *H. volcanii* WR441, lacking the *hdrA* gene encoding for DHFR-1 (24), were grown aerobically at 40°C (22). *H. volcanii* SX/CBD bearing the pWL-CBD plasmid encoding for the CBD moiety of the *C. thermocellum* cellulosome (25) was grown in the same medium to which novobiocin (1 µg/ml) was added. All strains were obtained from Moshe Mevarech (Tel Aviv University) and are characterized in Table 1.

Plasmid construction and transformation

Recombinant plasmids for expression of chimeric proteins bearing the SP of the *H. volcanii* SLG fused to either CBD from *C. thermocellum*, or to DHFR-1 from *H. volcanii* were constructed as follows (Fig 1A): The first 34 codons of the gene encoding for the *H. volcanii* SLG were PCR-amplified from plasmid pUC18-SLG (obtained from Manfred Sumper, University of Regensberg; 23) using the forward primer 5’-CTTGTCTAGACAGCTAA-3’ and reverse primer 5’-ATATCCATGCTGCGGCGC-3’, designed to introduce BspHI and NcoI
restriction sites at the 5'- and 3'-ends of the fragment, respectively. The PCR-amplified product was subcloned at the NcoI site of plasmid pWL-CBD, containing the *cbd* gene encoding the *C. thermocellum* cellulosome CBD fused to the haloarchaeal PrR16 promotor. This cloning yielded plasmid pWL-SP-CBD, containing a fusion of the 3'-end of the SLG signal peptide-encoding sequence ahead of the *cbd* gene, separated by a threonine-encoding codon linker, and behind the PrR16 promotor. The SP-encoding PCR-amplified fragment was also subcloned at the NcoI site of plasmid pHE1-DHFR-1 containing the *hdrA* gene (encoding *H. volcanii* DHFR-1) fused to the PrR16 promotor, to yield the pHE1-SP-DHFR-1 plasmid. *H. volcanii* cells were transformed with pWL-SP-CBD and pHE1-SP-DHFR-1 essentially as described (26) and selected in *H. volcanii* medium supplemented with novobiocin (1 µg/ml). The pWL-SP-CBD plasmid was used to transform *H. volcanii* WR 341 cells to create strain SX/SP-CBD, while pHE1-SP-DHFR-1 was used to transform *H. volcanii* WR441 to create strain WR441/SP-DHFR-1. Stable propagation of the plasmids was verified by plasmid reisolation and characterization by PCR analysis and DNA sequencing.

**Radiolabeling**

*H. volcanii* cells transformed with plasmid pWL–SP-CBD (30 ml) were grown to OD$_{590}$ = 0.6, harvested (8,000 x g, 15 min), resuspended in 30 ml of minimal medium (27) and grown aerobically at 40°C for 12 h. For pulse radiolabeling, [$^{35}$S] Met (143 µCi) was added to 10 ml of the cell culture, and 1 ml aliquots were removed at various intervals and immediately centrifuged (8,000 x g, 10 min, 4°C). The supernatant, containing the medium, was removed and kept on ice. The cells in the pellet were lysed with 1 ml of lysis buffer (1% Triton X-100, 1.8 M NaCl, 50 mM Tris-HCl, pH 7.2). For pulse chase radiolabeling, [$^{35}$S] Met (143 µCi) was added to 10 ml cultures in
minimal medium for 3-5 min, after which time unlabeled methionine was added to a final concentration of 1 mM. In some cases, anisomycin (20 µg/ml) was added during the final minute of radiolabeling. Aliquots were removed immediately prior to and at various intervals following the addition of the unlabeled methionine and processed as above. In both labelling protocols, DNase (3 µg/ml) was then added to the fractions. The mixtures were rocked (10 min, RT), after which time 50 µl of 10% (w/v) cellulose were added. After a 60 min rocking at RT, the suspension was centrifuged (1,000 x g, 3 min), the supernatant was discarded and the cellulose pellet was washed with 2 M NaCl, 50 mM Tris-HCl, pH 7.2. This washing procedure was repeated twice. After the final wash, the cellulose beads were centrifuged (2,200 x g, 3 min), the supernatant was removed and the cellulose pellet was resuspended in 40 µl SDS-PAGE sample buffer. The samples were then boiled for 5 min and centrifuged (5,000 rpm, 5 min) to release any cellulose-bound proteins. The proteins from cytoplasmic and media samples captured and later released from the cellulose beads were examined by 15% SDS-PAGE and viewed by fluorography using Kodak X-Omat film or a Fuji phosphorimager.

Other methods

Cells were separated from growth medium by centrifugation (8,000 x g, 15 min). The supernatant, containing medium and secreted proteins, was removed. The pelleted cells were resuspended in fresh growth medium and again collected by centrifugation. This washing step was repeated twice. In some cases, the separated cells were treated with proteinase K (1 mg/ml final concentration, 30 min, 40°C), in the presence or absence of 1% Triton X-100. Subcellular fractionation was achieved by osmotic lysis of cells upon transfer into 1 ml water, addition of DNase (3 µg/ml) and separation of the soluble and membrane fractions by ultracentrifugation (Sorvall Discovery M120...
ultracentrifuge (S120ATS rotor, 73,000 rpm, 10 min, 4°C). Immunoblotting was performed using antibodies raised against the *C. thermocellum* CBD (obtained from Arie Admon, Technion Israel Technology Institute), against *H. volcanii* DHFR-1 (obtained from Moshe Mevarech, Tel Aviv University) or against the *H. volcanii* SLG (28). Antibody binding was detected using goat anti-rabbit horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence. Protein concentration was determined using Bradford reagent (BioRad, Hercules CA), with BSA as standard. Densitometry was performed using IPLab Gel software (Signal Analytics, Vienna VI). For amino acid sequencing, proteins were electrotransferred to polyvinylidene fluoride membranes and subjected to analysis at the protein sequencing facility at the Weizmann Institute of Science (Rehovot, Israel).
RESULTS

SP-bearing fusion proteins are detected inside transformed H. volcanii cells

To assess the relation between protein translation and translocation in Archaea, H. volcanii WR341 cells were transformed with plasmid pWL-SP-CBD, encoding for a chimeric protein comprised of the SP of the H. volcanii SLG fused to the cbd gene, encoding for the C. thermocellum cellulosome CBD (Fig 1B). The synthesis and secretion of the chimeric reporter preprotein SP-CBD (22.5 kDa) was assessed by separating the transformed cells from their growth medium and analyzing the total protein content of each fraction by SDS-PAGE. At the same time, cellular fractions and growth media of the background WR341 and CBD-expressing cells were similarly analyzed. Coomassie staining revealed the presence of a protein corresponding in size to CBD (18.5 kDa) only in the growth medium of the SP-CBD-transformed strain (Fig 2A, lane 3). To confirm the identity of the secreted protein as the processed form of the SP-CBD fusion, i.e. CBD, N-terminal sequencing was performed. Such analysis revealed the first 6 amino acids as TMANTP, corresponding to the first five amino acid of CBD preceded by the additional threonine residue introduced during the cloning of plasmid pWL-SP-CBD (see Materials and Methods). Examination of the protein profiles of the cellular fractions of the three strains failed to reveal any visible differences (Fig 2A, lanes 4-6), indicating that the presence of mature CBD in the medium of SP-CBD-expressing cells is not the result of medium-contamination by ruptured cells in which accumulation or over-expression of the SP-CBD precursor had occurred. Thus, the results show that the SP-CBD-transformed strain successfully expresses and secretes the SP-CBD preprotein. At some point in the translocation event, the SP likely undergoes signal peptidase-mediated cleavage.
To confirm that CBD was only secreted from those cells transformed to express the SP-CBD chimera, background, CBD- and SP-CBD-expressing cells and their growth media were separated and subjected to immunoblotting using antibodies raised against CBD. As observed upon Commassie staining (Fig 2A), the antibodies only detected CBD protein in the growth medium of that strain transformed to express the SP-CBD fusion protein (Fig 2B, lanes 3). Immunoblotting of the cellular fractions of the three cultures also revealed the presence of the 18.5 kDa CBD in the CBD-expressing cells (Fig 2B, lane 5); no such band was stained in the non-transformed cells (Fig 2B, lane 4). Surprisingly, however, three bands were also recognized by the antibodies in the cellular fraction of the SP-CBD-expressing cells (Fig 2B, lane 6). The uppermost of these bands corresponds in size to the SP-fused version of CBD (full arrow). This suggests that SP-CBD is first completely synthesized inside the cells and only then secreted across the plasma membrane into the growth medium. The two additional smaller bands found inside the SP-CBD-transformed cells apparently represent partial breakdown products of the preprotein, likely resulting from proteolytic cleavage of the preprotein inside the cell, in agreement with earlier studies showing proteolysis of heterologously-expressed fusion proteins in *H. volcanii* (22, 29, 30). Subcellular fractionation of the SP-CBD-expressing cells revealed that the preprotein and its derived breakdown products were restricted to the cytoplasmic fraction (not shown).

To verify that the presence of SP-bearing precursor in transformed *H. volcanii* cells was not related to the use of a non-haloarchaeal protein entity such as CBD, *H. volcanii* cells were transformed with plasmid pHE1-SP-DHFR-1, encoding for a chimera of the *H. volcanii* SLG signal peptide fused to *H. volcanii* DHFR-1, one of the two versions of the cytoplasmic enzyme in this species (24). Expression of the chimeric preprotein in the transformed cells was verified by immunoblotting of
background, WR441 and WR441/SP-DHFR-1 strains using anti-DHFR-1 antiserum. The antibodies only recognized a protein band corresponding in size to DHFR-1 (17.8 kDa) in the growth medium of the WR441/SP-DHFR-1 culture (Fig 2C, lane 3). This indicates that *H. volcanii* cells are capable of secreting a normally cytoplasmic protein to which the SP of the SLG has been attached. In addition, the antibodies also recognized a slightly smaller band, likely a degradation product of DHFR-1. Next, the cellular fraction of each culture was probed with the DHFR-1 antibodies. As expected, DHFR-1 was stained by the antibodies in the control WR341 cells (Fig 2C, lane 4), yet was not detected in *H. volcanii* WR441, a DHFR-1-deletion strain (Fig 2C, lane 5). Examination of the cellular fraction of the SP-DHFR-1-transformed cells revealed the presence of the SP-bearing DHFR-1 preprotein (21.8 kDa), as well as a few smaller bands (Fig 2C, lane 6). Moreover, as is the case with SP-CBD-transformed cells, the presence of cellularly-associated, smaller anti-DHFR-1 antibody-labeled bands indicates that proteolytic cleavage of the preprotein also occurs inside the cell. Given the higher degree of SP-DHFR-1 breakdown as compared to SP-CBD degradation, reduced amounts of secreted, mature DHFR-1 would be expected. Accordingly, Coomassie staining failed to reveal secreted DHFR-1 in the growth medium of WR441/SP-DHFR-1 cells (data not shown).

**Cell-associated SP-CBD and SP-DHFR-1 are not externally accessible**

Experiments were next undertaken to confirm that the existence of cell-associated SP-bearing CBD and DHFR-1 preproteins was not due to small amounts of contaminating growth medium containing unprocessed secreted forms of the chimeric proteins being captured along with the isolated cells. In these studies, isolated SP-CBD-expressing cells were challenged with proteinase K so as to digest any SP-bearing CBD possibly present in medium that may have been captured along with the
isolated cells. As reflected in Fig 3 (upper panel), the protease treatment failed to affect SP-CBD levels, as detected by antibody staining (compare lanes 1 and 2). If, however, cell integrity was disrupted by pre-treatment with 1% Triton X-100, thereby allowing access of the protease to the cell’s interior, the SP-CBD precursor was completely digested (lane 3). The level of the SP-CBD-derived degradation product was relatively unaffected by the protease treatment, most likely owing to a protease-resistant conformation assumed by this polypeptide (31). Protease treatment also failed to affect levels of cell-associated SP-DHFR-1 or its breakdown products, as detected by antibody staining (Fig 3, middle panel, compare lanes 1 and 2). Inclusion of 1% Triton X-100 in the reaction, however, lead to a complete digestion of both the SP-DHFR precursor and its breakdown products (middle panel, lane 3). Finally, in control experiments, the ability of proteinase K to digest externally-oriented *H. volcanii* cell surface markers such as the SLG was confirmed in both WR441/SP-DHFR-1 cells (Fig 3, lower panel) and in WR341/SP-CBD cells (data not shown).

*Full-length nascent SP-CBD is first synthesized inside the cell and only then secreted*

Whereas full-length SP-bearing precursor proteins can be detected inside transformed cells, it is possible that these polypeptides correspond to translocation-incompetent preproteins that cannot be secreted and hence become entrapped within the cell. To discount this possibility, a series of kinetic, radiolabel-based studies were undertaken.

In pulse labeling experiments, SP-CBD-expressing cells were [*S]* metabolically radiolabeled. Samples were removed at various intervals and the labeled cells were separated from their growth media. Subsequently, the growth medium and solubilized cellular fractions were incubated with cellulose. Cellulose interacts with CBD in a
salt-independent manner (22), thereby allowing for affinity-based precipitation of the radiolabeled SP-CBD and of mature, secreted CBD in the high salt conditions required for *H. volcanii* growth. As shown in Fig 4A, analysis of cellulose-captured proteins by SDS-PAGE and fluorography first revealed the capture of the SP-CBD precursor in the cellular fraction close to the onset of the radiolabeling period. SP-CBD-derived breakdown products were also captured by cellulose in the cellular fractions. In contrast, the mature, processed form of the protein only appeared in the medium following a delay of 3 minutes.

The delay between the appearance of the full-length SP-CBD precursor in the cytoplasm and the detection of processed CBD in the growth medium could reflect the post-translational translocation of the preprotein. Alternatively, it is possible that a low efficiency of SP-CBD secretion would require that sufficient levels of CBD accumulate in the growth medium before detection of the secreted protein would be possible. In this latter scenario, translocation need not occur subsequent to translation, but rather could occur concomitantly. To discount this possibility, SP-CBD-transformed cells were subjected to pulse chase radiolabeling. Here, cells were metabolically radiolabeled with \[^{35}\text{S}\] methionine for 3 min following which time a large excess of unlabeled methionine was added to initiate the chase phase of the reaction. Aliquots were removed at various intervals and processed as above.

Examination of the cellular fractions revealed that the level of SP-CBD preprotein substantially decreased following the onset of chase (Fig 4B and 4C). Over the same period, an increase in the level of mature CBD in the growth media was measured. Thus, the pulse chase radiolabeling studies support the view that post-translation translocation of the SP-bearing CBD occurs in the transformed *H. volcanii* cells. The cellular fraction also contained a substantial amount of SP-CBD-derived breakdown
products, in agreement with earlier studies addressing the expression of fusion proteins by *H. volcanii* (29,32).

To further confirm that secretion of SP-CBD is temporally distinct from the translation of the protein, an additional experiment was performed in which pulse chase radiolabelling was repeated, however now, 20 µg/ml anisomycin were added during the final minute of the pulse phase of the experiment. Anisomycin is an effective inhibitor of protein synthesis in *H. volcanii* (14). Indeed, preliminary studies revealed that at the anisomycin concentration employed in these studies, protein synthesis in the SP-CBD-transformed cells was no longer detectable even as soon as 30 sec following exposure to the antibiotic (Fig 5A). Treatment with the antibiotic, however, had no effect on SP-CBD secretion since, as reflected in Fig 5B, radiolabeled mature CBD could be readily detected in the growth medium of the anisomycin-treated, preprotein-expressing cells. Moreover, anisomycin treatment did not prevent the rapid drop in cellular SP-CBD levels. Quantitation of mature CBD levels in the growth medium from three separate experiments confirmed that protein secretion continued well after the arrest of translation by the antibiotic (Fig 5C). These results confirm, therefore, that SP-CBD is first translated inside the cell and only then translocated across the plasma membrane into the growth medium, with SP cleavage occurring along the way.
DISCUSSION

In Archaea, a variety of proteins must be translocated into and across the plasma membrane, such as membrane proteins, secreted enzymes and the components of the protein-based surface-layer. Presently, numerous aspects of the archaeal protein export process remain to be elucidated, including whether archaeal translocation occurs post-translationally, as in Bacteria, or co-translationally, as in Eukarya. To investigate this question, *H. volcanii* cells were transformed to express chimeric preproteins formed from the SP of the *H. volcanii* SLG fused either to the CBD of the *C. thermocellum* cellusome, thus allowing for salt-insensitive, cellulose-based purification of the preprotein and its secreted product, or to *H. volcanii* DHFR-1, so as to monitor the secretion of a haloarchaeal cytoplasmic protein now designed for export. The transformed cells effectively expressed and secreted the chimeric preproteins, as detected by the presence of SP-cleaved versions of CBD and DHFR-1 in the respective growth media. However, examination of the protein content of the isolated cells revealed the presence of the full-length preproteins, suggesting that secretion of both the foreign CBD and the native DHFR-1 had occurred post-translationally. Kinetic radiolabeling experiments confirmed that secretion indeed took place only after the chimeric preproteins had been translated in the cytosol of transformed *H. volcanii* cells. Furthermore, arrest of continued protein translation had no effect on the secretion of previously pulse radiolabeled SP-CBD.

An alternative conclusion that might be drawn from these experiments is that the observed post-translation secretion reflects the cell’s efforts to cope with massive over-expression of the plasmid-encoded fusion proteins. This scenario, however, is unlikely due to several reasons. The plasmid employed for transformation of *H. volcanii* cells includes the non-inducible, constuitive PrR16 promotor (29,32).
Accordingly, no differences were noted in the growth rates or cell yields of the background and recombinant strains, effects that would be expected upon massive protein over-production. Indeed, Coomassie-staining of the cellular protein contents in Fig 2A failed to reveal strongly over-expressed proteins migrating at the positions of CBD or SP-CBD in the transformed \textit{H. volcanii} cells, as compared to the non-transformed background strain. Moreover, densitometric comparison of the intensities of anti-DHFR-1 antibody-reactive bands in background and SP-DHFR-1-transformed strains (Fig 2C) reveals that the transformed cells contain only 5-fold more immunoreactive material than the background cells. This value is likely an overestimate, given that it also includes the contribution of SP-DHFR-1 breakdown products that accumulate inside the cell and would not be secretion-competent. Thus, like earlier studies relying on this vector that failed to report large-scale over-expression of encoded proteins (22,29), it appears that massive over-expression of the fusion proteins is not the case in the present study. As such, it is unlikely that massively over-expressed SP-CBD and SP-DHFR-1 saturate a normally-employed co-translational secretion pathway and are thus secreted following translation. Indeed, regardless of the level of expression of the fusion proteins, the observation that protein secretion occurs at similar levels in the presence or absence of an inhibitor of haloarchaeal protein synthesis offers strong support that the post-translational secretion observed in these studies reflects a normal and constitutive cellular process.

The SP of the \textit{H. volcanii} SLG employed to target CBD and DHFR-1 for export in the present study displays a molecular composition similar to that borne by SPs of Sec-dependent preproteins in Eukarya and Bacteria (3). Given the similarity of SP composition, it is reasonable to predict that the archaeal preproteins in this study also rely on the Sec machinery for their secretion. In Eukarya, the vast majority of
secretory proteins are co-translationally targeted to Sec translocation sites in the ER membrane, i.e. the translocon, via the SRP pathway (15). Despite the fact that the archaeal SRP is, in many ways, similar to its eukaryal counterpart (16), it seems that archaeal protein secretion shares more in common with Bacteria, the other prokaryal domain, as both Bacteria and Archaea apparently rely on a post-translational mode of protein secretion.

In the bacterial Sec-pathway, SecB or other chaperones post-translationally deliver secretory protein precursors to SecA, peripherally-bound to the membrane at translocation sites based on the trimeric SecYEG complex (11,33). Exploiting its ATP hydrolyzing activity, SecA then drives the transfer of its preprotein cargo across the plasma membrane. Whereas Archaea encode for versions of SecYEG (13,14,34) (and possibly SecB (13)), searches of completed archaeal genomes have yet to detect an archaeal SecA homologue. The existence of a structural homologue of SecA in Archaea, not detectable by current bioinformatic approaches, cannot, however, be ruled out. It is noteworthy, therefore, that despite the apparent absence of SecA in Archaea, several archaeal species encode for homologues of SecDF (17), components shown to modulate the membrane association of SecA in E. coli (18,19).

Examples of post-translational, SecA-independent translocation via the Sec-pathway do exist. In yeast, post-translational protein export relies on various cytoplasmic chaperones for the delivery of preproteins to the translocon (35). Once delivered, the ER resident protein BiP employs ATP energy to pull the preprotein into the ER lumen (36,37). It is unlikely that Archaea would rely on a similar mechanism, given the improbability of nucleotide being present in sufficient quantities on the exterior surface of the plasma membrane, the topological homologue of the lumenal face of
the ER membrane. Moreover, many of the chaperones involved in eukaryal post-translational translocation are absent in several archaeal species (38). As such, components involved in the post-translational targeting and translocation of secretory proteins across the archaeal plasma membrane may remain to be identified. With this in mind, it is noteworthy that two recent studies have reported extensive use of the alternative Tat secretion pathway, involved in post-translocational translocation in Bacteria, in haloarchaea (39, 40).

The successful ability of the fused *H. volcanii* SLG signal peptide to target both CBD and DHFR-1 to the growth medium may reveal other general aspects of the translocation process in this species and possibly in other Archaea. The *H. volcanii* SLG, the sole component of the proteinaceous shell surrounding the cell, spans the plasma membrane via a single transmembrane domain (23). Thus, the finding that the same SP can direct both a membrane-anchored protein as well as secreted proteins to the cell exterior suggests that both classes of proteins rely on the same translocation apparatus.

Finally, although only a very limited number of cases have been addressed, it is tempting to speculate that in Archaea, protein secretion occurs post-translationally while membrane protein insertion occurs in an SRP-dependent, co-translational manner. Not all archaeal membrane proteins, however, may rely on the SRP-pathway for their insertion, as possibly exemplified by studies address the homologous (20, 41) and heterologous (22) expression of bacterioopsin. Indeed, the SRP pathway is implicated in the membrane insertion of only a subset of bacterial plasma membrane proteins (42). Given the availability of inverted membrane vesicles from *H. volcanii* (43), reconstituted *H. volcanii* SRP (44) purified SP-CBD\(^2\) and SP-bacterioopsin-
DHFR-1 (22) preproteins, the stage is now set to reconstitute archaeal protein translocation \textit{in vitro} and address these questions.
ACKNOWLEDGEMENTS

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REFERENCES


FOOTNOTES

1. Abbreviations used in the text: CBD, cellulose binding domain; DHFR, dihydrofolate reductase; SLG, surface layer glycoprotein; SRP, signal recognition particle; SP, signal peptide

2. V.I. and J.E., unpublished observations
TABLE 1: Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
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<td>pWL-DHFR-1</td>
<td>pWL-Nov vector containing the PrR16 promotor fused to the <em>H. volcanii</em> <em>hdrA</em></td>
<td>24</td>
</tr>
<tr>
<td>pWL-SP-CBD</td>
<td>pWL-CBD bearing the <em>H. volcanii</em> SLG SP fused to CBD.</td>
<td>This study</td>
</tr>
<tr>
<td>pHE1-SP-DHFR-1</td>
<td>pHE1-DHFR-1 bearing the <em>H. volcanii</em> SLG SP fused to DHFR-1.</td>
<td>This study</td>
</tr>
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</table>
FIGURE LEGENDS

Fig 1 – A. Schematic representations of plasmids pWL-SP-CBD and pHE1-SP-DHFR. See Materials and Methods for description of the plasmids and their construction. B. Schematic representations of SP-CBD and SP-DHFR-1. The amino acid sequences of the SP of the SLG and the first 10 amino acid residues of the CBD and DHFR-1 moieties are shown. The position of the signal peptidase cleavage site is marked, as is the position of the addition threonine residue introduced during construction of the plasmids. In addition, the basic (n-), hydrophobic (h-) and polar cleavage (c-) regions of the SP are indicated.

Fig 2 – SP-bearing preproteins are detected in the cytosol while SP-cleaved proteins are found in the medium. A. Aliquots (1 ml) of cultures of *H. volcanii* (WR 341), and *H. volcanii* transformed to express either CBD or SP-CBD were separated into cellular and growth media fractions and each was examined by SDS-PAGE. Molecular weight markers are shown on the right of each panel, with the value of each marker only shown for the cells panel. To the right of the media panel, the full arrow reflects the expected position of SP-CBD while the open arrow reflects the position of CBD. B. The separated cellular and media fractions of 200 µl aliquots of each cell culture were probed with antibodies to CBD. The full arrow reflects the position of SP-CBD while the open arrow reflects the position of CBD. The positions of molecular weight markers are shown on the right. C. Aliquots (200 µl) of cultures of *H. volcanii* (WR341), *H. volcanii* lacking the gene encoding DHFR-1 (WR441) and WR441 transformed to express DHFR-1 (WR 441/SP-DHFR-1) were separated into cellular and growth media fractions and each was electroblotted and probed with antibodies to DHFR-1. The full arrow reflects the position of SP-DHFR-1 while the open arrow reflects the position of DHFR-1.
Fig 3 – SP-bearing preproteins are protected from added protease. *H. volcanii* cells transformed to express SP-CBD (upper panel), SP-DHFR-1 (middle and lower panels) were exposed to the presence of proteinase K in the absence (lane 2) or presence (lane 3) of 1% Triton X-100. In lane 1, neither protease nor detergent was added. The samples were then subjected to immunoblotting with antibodies against the CBD (upper panel), DHFR-1 (middle panel) or the SLG (lower panel). The positions of SP-CBD, SP-DHFR-1 and the SLG are denoted in the upper, middle and lower panels, respectively.

Fig 4 – SP-CBD is first translated in the cytosol and then secreted into the growth medium. *H. volcanii* cells transformed to express SP-CBD were metabolically [³⁵S] pulse radiolabeled or radiolabeled in a pulse chase paradigm. Aliquots were removed at various intervals and transferred to ice-cold tubes. Cellular and media fractions were separated, incubated with cellulose and examined by SDS-PAGE and fluorography. A. Pulse radiolabeling. B. Pulse chase radiolabeling. In both A and B, the position of SP-CBD is denoted by the full arrow while the position of CBD is denoted by the open arrow. C. The fluorograph of the pulse chase radiolabeling was densitometrically analyzed to determine the intensity of the SP-CBD (full circles) and CBD (open circles) bands.

Fig 5 - Arrest of continued protein translation does not interfere with SP-CBD secretion. A. *H. volcanii* cells were [³⁵S]-metabolically radiolabeled (15 µCi/ml, 5 min) before or after pretreatment with anisomycin (20 µg/ml) for varying lengths of time. At each time point, the samples were TCA-precipitated and examined by SDS-PAGE and fluorography. The fluorograph was purposely over-exposed so as to
confirm the inhibition of protein translation by the antibiotic. The positions of molecular weight markers (250, 150, 100, 75, 50, 37, 25, 15 and 10 kDa) are shown on the right. B. Anisomycin (20 µg/ml) was added to SP-CBD transformed H. volcanii cells during the final minute of a 5 min [35S] radiolabelling pulse. Chase was then initiated upon addition of an excess of unlabeled methionine. Aliquots were removed either immediately before or at various intervals following the onset of the chase. Cells and growth medium were isolated from each time point, incubated with cellulose and examined by SDS-PAGE and fluorography. C. Densitometric quantitation of the level of CBD secreted into the growth medium. The values, expressed as fold-increase from the onset of chase (taken as 1.0), represent the average of 2-3 experiments ± standard deviation.
A.

ampicillin<sup>R</sup>  \[ \text{E. coli ori} \]

H. volcanii ori  \[ \text{novobiocin}^R \]

Pr16  SP  CBD
Pr16  SP  DHFR

BamHI  Ncol  Ncol  XbaI

B.

n-region    h-region    c-region

MTKLKDQTRAILLATLMVTSMVFAGAIATFGSAAA

TMATPVSGLNL
TMELVSSVAALA

SLG signal peptide

cleavage site

introduced residue

CBD

DHFR-1

Irihimovitch and Eichler, Fig 1
Irihimovitch and Eichler, Fig 2
Irihimovitch and Eichler, Fig 3

- SP-CBD
- SP-DHFR
- SLG

proteinase K: -  +  +  +
Triton X-100: -  -  +  +
A. anisomycin pre-treatment (min): 0 0.5 1

B. pulse (5 min) → chase (min): anisomycin (1 min) → 0 1 3 5 10 15

C. fold increase vs. time (min)

Irihimovitch and Eichler, Fig 5
Post-translational secretion of fusion proteins in the halophilic archaeon Haloferax volcanii
Vered Irihimovitch and Jerry Eichler

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