

In the Archaea *Haloferax volcanii*, Membrane Protein Biogenesis and Protein Synthesis Rates Are Affected by Decreased Ribosomal Binding to the Translocon*

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In the haloarchaea *Haloferax volcanii*, ribosomes are found in the cytoplasm and membrane-bound at similar levels. Transformation of *H. volcanii* to express chimeras of the translocon components SecY and SecE fused to a cellulose-binding domain substantially decreased ribosomal membrane binding, relative to non-transformed cells, likely due to steric hindrance by the cellulose-binding domain. Treatment of cells with the polypeptide synthesis terminator puromycin, with or without low salt washes previously shown to prevent *in vitro* ribosomal membrane binding in halophilic archaea, did not lead to release of translocon-bound ribosomes, indicating that ribosome release is not directly related to the translation status of a given ribosome. Release was, however, achieved during cell starvation or stationary growth, pointing at a regulated manner of ribosomal release in *H. volcanii*. Decreased ribosomal binding selectively affected membrane protein levels, suggesting that membrane insertion occurs co-translationally in Archaea. In the presence of chimera-incorporating sterically hindered translocons, the reduced ability of ribosomes to bind in the transformed cells modulated protein synthesis rates over time, suggesting that these cells manage to compensate for the reduction in ribosome binding. Possible strategies for this compensation, such as a shift to a post-translational mode of membrane protein insertion or maintained ribosomal membrane-binding, are discussed.

Although proper protein localization is essential for the survival of all cells, various strategies exist for delivering proteins across membranes. In Eukarya, protein transfer across the endoplasmic reticulum (ER)¹ membrane occurs in a co-translational manner (1). Here, ribosomes translating nascent polypeptide chains destined for transfer across the ER membrane are delivered to the translocon, the Sec61 $\alpha\beta\gamma$ -based membrane protein complex that serves as the site of translocation. Correct targeting of such ribosomes relies on the signal recognition particle (SRP) pathway, comprising the ribonucleoprotein SRP and its membrane-associated receptor (2). In

contrast, bacterial protein secretion is essentially a post-translational process, taking place once most, if not all, of a signal sequence-bearing nascent polypeptide chain has been translated by cytoplasmic ribosomes (3, 4). Through interactions with molecular chaperones such as SecB, the nascent preprotein is delivered to the SecYEG-based translocon, where SecA uses ATP energy to drive translocation across the plasma membrane. Examples of co- and post-translational translocation have, however, been reported in Bacteria and Eukarya, respectively. In Bacteria, the SRP pathway serves to target ribosomes in the process of translating membrane proteins to the translocon in a co-translational manner (5, 6), whereas in yeast, preproteins may be translocated post-translationally across the ER membrane at a translocon that also includes the tetrameric Sec62/63 complex (7).

In contrast to the well defined eukaryal and bacterial protein translocation systems, comparatively less is known of how proteins cross the membranes of Archaea, the third domain of life (8, 9). The solution of the three-dimensional structures of the *Haloarcula marismortui* ribosome large subunit (10), the *Methanocaldococcus* (*Methanococcus*) *jannaschii* SecYE β complex (11), and SRP components from various Archaea (12–14) suggests, however, that investigations into archaeal protein translocation will provide novel insight into the process across evolutionary lines. Toward this end, the relation between protein synthesis and translocation in Archaea has been the focus of several recent studies.

In the halophilic archaea *Haloferax volcanii*, secretion of signal sequence-bearing chimeric reporter proteins was shown to occur post-translationally (15), despite the fact that Archaea apparently fail to encode for a SecA homologue (8, 9). In contrast, the membrane insertion of *Halobacterium salinarum* bacterioopsin (Bop), the apoprotein form of the light-driven multispanning proton pump bacteriorhodopsin, relies on a co-translational process (16–18). The co-translational nature of Bop membrane insertion has been questioned, however, with the report of post-translational insertion of a Bop-containing fusion protein heterologously expressed in *H. volcanii* (19). As such, a dedicated Bop insertion system may exist in *H. salinarum*. Indeed, the Bop signal sequence is strikingly different from the standard signal sequences found in other membrane or secretory preproteins (16).

Nonetheless, co-translational translocation may be a general property of Archaea. Comprising an eukaryal-like SRP and a bacterial-like SRP receptor (20, 21), the archaeal SRP pathway could serve to target translating ribosomes to the membrane, as in Eukarya and Bacteria. To date, archaeal SRP from several species has been reassembled from purified components (22–24) and has been isolated from *H. volcanii* (25), whereas binding of the archaeal SRP receptor to membrane vesicles has also been shown (26, 27). Moreover, as in Eukarya (28, 29) and

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¹ The abbreviations used are: ER, endoplasmic reticulum; Bop, bacterioopsin; CBD, cellulose binding-domain; SLG, S-layer glycoprotein; SRP, signal recognition particle.

Bacteria (30, 31), recent *in vitro* studies have revealed that the translocon also serves as the membrane ribosomal receptor in Archaea (32). However, given current technical limitations, it is not yet possible to incorporate these steps into a reconstituted membrane protein insertion system.

With this in mind, a series of *in vivo* experiments was devised to address the relationship between protein synthesis and membrane protein insertion in *H. volcanii*. We report that *H. volcanii* membranes contain bound ribosomes at levels reminiscent of the eukaryal ER membrane and that neither puromycin nor low salt treatments lead to the release of membrane-bound *H. volcanii* ribosomes. Release readily occurred, however, following starvation or during stationary growth, pointing to ribosomal release being a regulated process in Archaea and not simply mediated by the protein synthesizing status of a given ribosome. We also show that the decreased ribosomal membrane binding that occurs in the presence of sterically hindered translocons in transformed *H. volcanii* cells leads to a selective decrease in nascent membrane protein integration, suggesting that in Archaea, membrane protein biogenesis occurs co-translationally. The decrease in the number of ribosome binding sites and subsequent effect on membrane protein insertion moreover served to modulate rates of protein synthesis in the transformed cells, relative to the background strain. As such, the results offer insight into the relation between ribosome binding, protein synthesis, and membrane protein insertion in Archaea.

EXPERIMENTAL PROCEDURES

Materials—Diethylpyrocarbonate, novobiocin, and puromycin came from Sigma. Molecular weight markers were obtained from Fermentas (Burlington, Canada). TRIzol reagent was purchased from Invitrogen. Horseradish peroxidase-conjugated goat anti-rabbit antibodies were purchased from Bio-Rad. An ECL enhanced chemiluminescence kit, as well as Redivue ^{35}S radiolabeling mixture (>1000 Ci/mmol), were obtained from Amersham Biosciences.

Subcellular Fractionation—*H. volcanii* background (WR341) and CBD-SecE- and CBD-SecY-expressing cells were grown in rich medium to mid-exponential or stationary phase and identical optical densities, pelleted at $9,000 \times g$ for 10 min, and disrupted by gentle lysis in lysis buffer (3.4 M KCl, 100 mM MgCl₂, 10 mM Tris-HCl, pH 7.6) according to Gropp *et al.* (16). After a 1-h incubation at room temperature, the cell lysate was separated into cytoplasmic and membrane fractions by ultracentrifugation (Sorvall Discovery M120 ultracentrifuge, S120AT2 rotor, $196,000 \times g$, 10 min, 4 °C). The cytoplasmic and membrane proteins were analyzed by SDS-PAGE and immunoblotting using antibodies raised against *H. volcanii* S-layer glycoprotein (SLG) (33), *H. volcanii* dihydrofolate reductase-1 (obtained from Moshe Mevarech, Tel Aviv University, Tel Aviv, Israel) or against *H. marismortui* ribosomal L11 protein (obtained from Ada Yonath, Weizmann Institute of Science, Rehovot, Israel). Alternatively, the rRNA content of the cytoplasmic and membrane fractions was examined.

RNA Extraction—RNA was extracted with TRIzol, according to the manufacturer's instructions. Fractions of background and CBD-SecE- and CBD-SecY-expressing cells grown to identical optical densities were extracted with TRIzol and chloroform, after which the RNA was precipitated with isopropanol. The RNA pellet was washed with 75% ethanol and dissolved in diethylpyrocarbonate-treated water. After addition of loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol, 95% formamide, 20 mM EDTA-KOH, pH 8.0), the samples were examined by electrophoresis on 1.5% denaturing agarose gels (34).

Gradient Floatation Analysis—Gradient floatation analysis was performed as described previously (32). Briefly, the membrane fraction achieved after cell fractionation was transferred to the bottom of a S120AT2 rotor tube and mixed with 400 μl of buffer F1 (1.7 M sucrose in lysis buffer). The sample was then overlaid with 600 μl of buffer F2 (1.5 M sucrose in lysis buffer) and 300 μl of lysis buffer. After ultracentrifugation (100,000 rpm, 2 h, 4 °C), six 200- μl aliquots were collected from the top of each gradient down. The protein and RNA contents of the aliquots were analyzed as described above.

Metabolic ^{35}S Radiolabeling—Background and CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells were grown in rich medium to mid-exponential phase and transferred to minimal medium containing all

amino acids (19). After a 24-h incubation at 40 °C, the cells were pelleted and transferred to cysteine/methionine-lacking minimal medium for an additional 90-min incubation. Equal amounts of the cells were then radiolabeled with [^{35}S]cysteine/methionine (14 $\mu\text{Ci/ml}$) for 1 h and subjected to subcellular fractionation. The level of radioactive protein present in both the soluble and membrane fractions of each cell type was then determined by precipitation onto filters and scintillation counting in a β -counter or by SDS-PAGE electrophoresis and fluorography using Kodak BioMax XAR film.

Puromycin Treatment—Puromycin treatment was performed as described previously (16), with slight modifications. *H. volcanii* cells were grown to mid-exponential phase and incubated with or without puromycin at a final concentration of 70 $\mu\text{g/ml}$. After a 20-min incubation at 40 °C, the cells were pelleted and resuspended in lysis buffer. Following subcellular fractionation as described above, the RNA content of the cytoplasmic and membrane fractions was extracted and examined by electrophoresis on 1.5% denaturing agarose gels.

Other Methods—Antibody binding was detected using goat anti-rabbit horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence. Densitometry was performed using IPLab Gel software (Signal Analytics, Vienna, VA). Immunoblots in the linear response range were used for densitometric quantitation.

RESULTS

In *H. volcanii*, a Major Proportion of Ribosomes Are Membrane-bound at Sec Translocons—Earlier *in vitro* studies reported the binding of *H. volcanii* ribosomes to the membrane at SecYE-based translocon sites (32). In the present report, the *in vivo* binding behavior of *H. volcanii* ribosomes was considered. As a first step, the extent of ribosomal membrane binding in the cell was addressed. Accordingly, *H. volcanii* cells were grown to mid-exponential phase, harvested, and disrupted by gentle lysis. Following separation of the cytoplasmic and membrane fractions by ultracentrifugation, the efficiency of subcellular fractionation was confirmed by assessing the distribution of the SLG, a marker of the *H. volcanii* membrane, and dihydrofolate reductase-1, a marker of *H. volcanii* cytosol, by immunoblotting (not shown). The presence of ribosomes in each subcellular fractionation was next determined by testing for the presence of 23 S and 16 S rRNA, as well as L11 protein, following RNA extraction or immunoblotting, respectively. As reflected in Fig. 1A, both rRNA and protein markers were detected in each subcellular fraction. Densitometric analysis of the combined distribution of 23 S and 16 S rRNA revealed $58\% \pm 3.5$ ($n = 11$) of the cellular ribosomal content to be membrane-associated, whereas similar quantitation of the distribution of L11 revealed $42\% \pm 3.5$ ($n = 11$) of the protein to be membrane-associated. To confirm the specific nature of the ribosome-membrane interaction, the isolated membrane fraction was subjected to floatation on sucrose gradients. Immunoblotting of the SLG in the six fractions collected from the centrifugation tube confirmed that the membranes had floated to the upper portion of the gradient. As reflected in the distribution of 23 S and 16 S rRNA and L11 protein in the gradients, ribosomes co-migrated with the membrane fragments (Fig. 1B). As such, the proportion of membrane-associated ribosomes in *H. volcanii* is far more similar to that reported in eukaryal cells (~60–80%) (35, 36) than what has been reported in Bacteria (~5–10%) (37, 38).

When the extent of ribosomal membrane binding was evaluated in cells grown to stationary phase, a much different picture was presented. In this case, a 35% decrease in total ribosome levels, relative to cells in exponentially growing cells, was observed, in agreement with earlier studies examining the relation between growth rate and ribosome content in halophilic archaea (39, 40). Moreover, the ribosomes displayed a very different distribution. In cells grown to stationary phase, $74\% \pm 1.4$ ($n = 4$) of the ribosomes, as detected by densitometric quantitation of rRNA levels, were found in the soluble fraction. When L11 protein levels were considered, immuno-

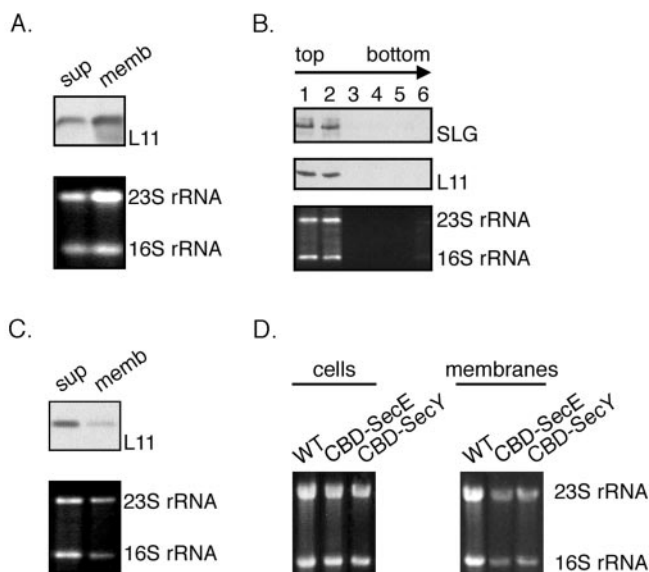


FIG. 1. Ribosomal membrane binding is significantly reduced in CBD-SecE- or CBD-SecY-expressing *H. volcanii* cells. *A*, a 1-ml aliquot of *H. volcanii* cells grown to mid-exponential phase ($A_{550} = 1.5$) was subjected to subcellular fractionation. The soluble (*sup*) and membrane (*memb*) fractions were then analyzed by immunoblotting using anti-L11 antibodies or for 23 S and 16 S rRNA following RNA extraction and electrophoresis on denatured agarose gels. *B*, the isolated membranes were subjected to floatation on sucrose density gradients as described under "Experimental Procedures." Six fractions were collected from the top of the gel and probed with antibodies against the *H. volcanii* SLG or L11, or for the presence of 23 S and 16 S rRNA, as above. *C*, as in *A*, except that cells grown to stationary phase ($A_{550} = 2.5$) were used. *D*, the ribosome content of background strain WR341 (WT) and CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells or isolated membranes from each strain was determined by comparing levels of 23 S and 16 S rRNA.

blotting and densitometry revealed that $78\% \pm 1.4$ ($n = 4$) of the protein could be detected in the soluble phase (Fig. 1C). Hence, during stationary growth, *H. volcanii* cells not only contain less ribosomes but also contain far fewer membrane-bound ribosomes than do cells in the exponential phase of growth.

Experiments aimed at defining the site of ribosomal membrane binding in the archaeal cells were next undertaken. Earlier *in vitro* studies had shown that inverted membrane vesicles prepared from *H. volcanii* cells transformed to express chimeras of either SecY or SecE fused to the cellulose binding-domain (CBD) of *Clostridium thermocellum* were significantly compromised in their abilities to bind externally added ribosomes, likely due to the steric hindrance provided by the CBD moiety at Sec sites (32). Accordingly, membranes were isolated from equivalent amounts of the *H. volcanii* WR341 background strain or the same *H. volcanii* cells transformed to express CBD-SecE and CBD-SecY (41), and the level of bound ribosomes was determined in each case. Given the similarities in growth rate (see below) and cell size (not shown) of the three cell types, analysis of equivalent volumes of cultures grown to identical optical densities allowed for direct comparison of the level of bound ribosomes in the different strains. Whereas the presence of CBD-SecE or CBD-SecY had no effect on the total number of ribosomes, as reflected in comparable total 23 S and 16 S rRNA levels (Fig. 1D, left panel), expression of the chimeras led to a substantial decrease in ribosomal membrane binding (right panel). Control experiments, in which the distribution of a membrane protein marker, the SLG, was considered, confirmed the efficiency of membrane recovery in each case as well as that these comparisons examined similar amounts of membranes from each strain (not shown). Thus, as observed *in*

vitro, *H. volcanii* ribosomes bind to the membrane at Sec sites *in vivo*, despite a haloarchaeal cytoplasmic salt concentration reported to be as high as 5 M KCl (42, 43).

***H. volcanii* Ribosomes Remain Membrane-bound following Puromycin and Low Salt Treatments**—A series of experiments examining the nature of the ribosome-translocon interaction in *H. volcanii* cells was next undertaken. To determine whether membrane-bound *H. volcanii* ribosomes remained associated with the translocon following the termination of protein synthesis, *H. volcanii* cultures were incubated with puromycin prior to cell harvesting and membrane isolation. In preliminary experiments, puromycin, an antibiotic that leads to premature release of nascent polypeptide chains from the ribosome, was shown to inhibit protein synthesis in *H. volcanii* after a 20-min incubation at a final antibiotic concentration of 70 $\mu\text{g/ml}$ (Fig. 2A). When *H. volcanii* cells were exposed to puromycin at levels sufficient to fully arrest continued protein synthesis, subcellular fractionation and subsequent analysis of the distribution of ribosomal large and small subunits, as reflected in 23 S and 16 S rRNA levels, respectively, presented a picture extremely similar to that obtained with untreated cells (Fig. 2B and Table I). Densitometric quantitation revealed that 93% of the membrane-bound 50 S subunit (as detected by 23 S rRNA levels) and 96% of the membrane-bound 30 S subunit (as detected by 16 S rRNA levels) remained membrane-associated after the puromycin treatment, relative to the amount of ribosomes bound to the membranes of untreated cells. As such, it appears that the puromycin-induced release of the nascent polypeptide chain from the ribosome, or by extension, termination of protein synthesis, was insufficient to cause ribosomes to detach from the translocon.

The effect of salt concentration on the release of bound *H. volcanii* ribosomes was next addressed. It had been previously shown that high salt levels were essential for the binding of purified, non-translating functional ribosomes to *H. volcanii* inverted membrane vesicles (32), as would be expected in a halophilic archaea with intracellular salt concentrations as high as 5 M (42, 43). In those *in vitro* studies, no ribosome binding to the membrane could be detected at salt concentrations lower than 2 M. Thus, to test whether low salt treatment could also strip prebound ribosomes, *H. volcanii* membranes were isolated and washed with buffer containing 1 M KCl, a salt concentration previously shown to prevent ribosome binding *in vitro* (32). When the isolated membranes were subjected to the low salt treatment, a substantial portion of bound ribosomes was released from the membrane. Densitometric analysis revealed that only 47% of the membrane-bound 50 S subunit (as detected by 23 S rRNA levels) and 30% of the membrane-bound 30 S subunit (as detected by 16 S rRNA levels) remained membrane-associated (Table I). It is likely that much of the ribosomal release is related to a loss of ribosomal protein structural integrity, as would be expected with halophilic proteins exposed to low salt conditions, rather than reflecting changes in membrane binding properties *per se*. Indeed, the increased loss of the 30 S subunit is probably due to low salt-induced dissociation of the small subunit from intact, membrane-bound 70 S ribosomes. Thus, whereas exposure to low salt led to more ribosomal release than puromycin-induced translation termination, ~50% of the membrane-bound ribosome 50 S subunit was not released by washes with low salt. This is in contrast to the *in vitro* situation, where low salt prevented any *H. volcanii* ribosome binding (32).

Next, the combined effects of low salt and termination of protein synthesis on ribosome release were addressed. Accordingly, membranes from *H. volcanii* cells grown in the presence of puromycin were isolated and washed with buffer containing

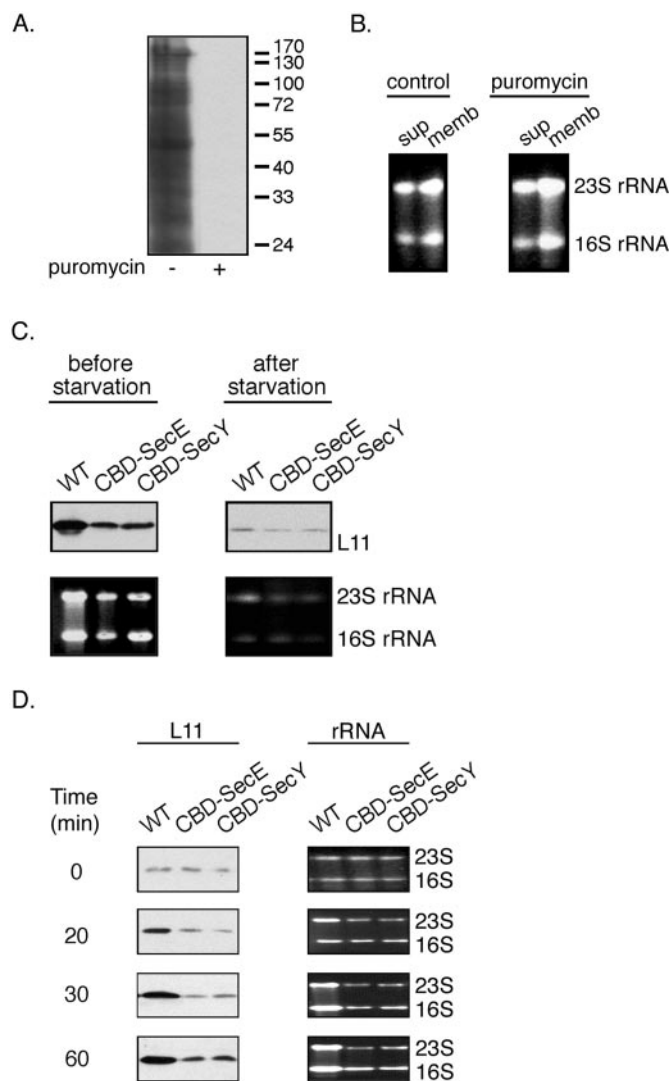


FIG. 2. Puromycin treatment does not lead to release of membrane-bound ribosomes in *H. volcanii*, but starvation does. A, *H. volcanii* cells were incubated in the absence or presence of 70 $\mu\text{g/ml}$ puromycin 20 min prior to radiolabeling with [^{35}S]cysteine/methionine radiolabeling mixture (14 $\mu\text{Ci/ml}$) for 5 min. Aliquots were then subjected to SDS-PAGE and fluorography. The positions of molecular weight markers are shown on the left. B, *H. volcanii* cells incubated in the absence or presence of 70 $\mu\text{g/ml}$ puromycin for 20 min were subjected to subcellular fractionation and the distribution of 23 S and 16 S rRNA, extracted, and examined as described under "Experimental Procedures," in the soluble (*sup*) and membrane (*memb*) fractions was determined. C, the ribosome content of the membrane fraction of background (WT) and CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells was determined by immunoblotting with anti-L11 antibodies or by analysis of 23 S and 16 S rRNA following RNA extraction and electrophoresis on denatured agarose gels before or after a 90-min transfer to cysteine- and methionine-lacking minimal medium. D, background (WT) and CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells were grown to $A_{550} = 1.5$ at which time they were transferred to minimal medium lacking both cysteine and methionine. 90 min later, cysteine/methionine-containing medium was added, and aliquots removed at the indicated times. The membrane fraction of each aliquot was then processed for immunoblotting with anti-L11 antibodies (left) or for rRNA extraction (right), as above.

1 M KCl. Densitometric analysis revealed that 49% of the membrane-bound 50 S subunit (as detected by 23 S rRNA levels) and 29% of the membrane-bound 30 S subunit (as detected by 16 S rRNA levels) remained membrane-associated, relative to the amount of ribosomes bound to the membranes of untreated cells (Table I). Thus, while treatment with puromycin followed by low salt washes led to considerably more ribosomal detach-

TABLE I
Ribosomal binding to *H. volcanii* membranes following various treatments

Treatment	50 S subunit ^a	30 S subunit ^b
Control	100 ^c	100
Puromycin	93 \pm 0.4 (5)	96 \pm 0.4 (5)
Low salt	47 \pm 3 (5)	30 \pm 4 (5)
Low salt plus puromycin	49 \pm 7 (5)	29 \pm 5 (5)

^a As detected by 23 S rRNA levels.

^b As detected by 16 S rRNA levels.

^c Values are expressed as percentage \pm S.D. (number of repeats), with the densitometrically determined levels of membrane-bound ribosomes in exponentially growing cells taken as 100%.

ment from the membrane than when the antibiotic was used alone, the combination of treatments did not release more ribosomes than when low salt was used alone. The inability of the puromycin and low salt treatments, separately or together, to fully release membrane-bound *H. volcanii* ribosomes could reflect membrane release being a regulated process rather than simply relying on the translation status of the ribosome.

A far greater extent of ribosomal release was achieved when *H. volcanii* cells were grown for 90 min in a starvation minimal medium lacking cysteine/methionine, as used prior to metabolic ^{35}S radiolabeling. Fig. 2C (left panels) reveals that, prior to transfer into the starvation medium, ribosomal membrane binding was readily detected in the background strain as well as in CBD-SecE- and CBD-SecY-expressing cells, albeit to a much lesser degree (in agreement with the results presented in Fig. 1D). Following a 90-min incubation in the starvation medium, during which time a 39% reduction in total ribosome levels took place, a drastic drop in the fraction of membrane-bound ribosomes was detected in all three *H. volcanii* strains (Fig. 2C, right panels). Indeed, the level of membrane-bound ribosomes was reminiscent of that observed on the membranes isolated from *H. volcanii* cells grown to stationary phase (compare Figs. 1C and 2C). Upon subsequent transfer of the starved cells into cysteine/methionine-containing minimal medium, binding of ribosomes to the membrane could be observed in both the background and the transformed cells. Indeed, ribosomal binding to the degree observed in exponentially growing cells could be detected 1 h after transfer into cysteine/methionine-containing medium, with recovery of ribosome binding occurring much more rapidly in the background than in the chimera-expressing cells (Fig. 2D). The degree of ribosomal binding achieved during both stationary growth and upon starvation, may indicate that release of membrane-bound ribosomes occurs in a regulated manner, related to the physiological status of the cell.

Translocon-bound *H. volcanii* Ribosomes Are Involved in Membrane Protein Biogenesis—The effect of decreased ribosomal membrane binding in CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells on protein biogenesis was next considered. In these experiments, the background and chimera-expressing cells were transferred to cysteine/methionine-free minimal medium for 90 min and then metabolically labeled with [^{35}S]cysteine/methionine for 60 min, after which time the cells were subjected to subcellular fractionation. The level of radioactive protein present in both the soluble and membrane fractions of each cell type was then determined by precipitation onto filters and scintillation counting. Analysis of the radioactivity incorporated into nascent cytoplasmic polypeptides revealed that the presence of CBD-SecE or CBD-SecY had only minor effects on soluble protein synthesis (Fig. 3A). Specifically, only an \sim 10% drop in the level of cytoplasmic protein synthesis was detected in CBD-SecE-expressing cells, relative to the background strain. Similarly, the level of *de novo* soluble protein synthesis was only under 20% lower in CBD-SecY-expressing

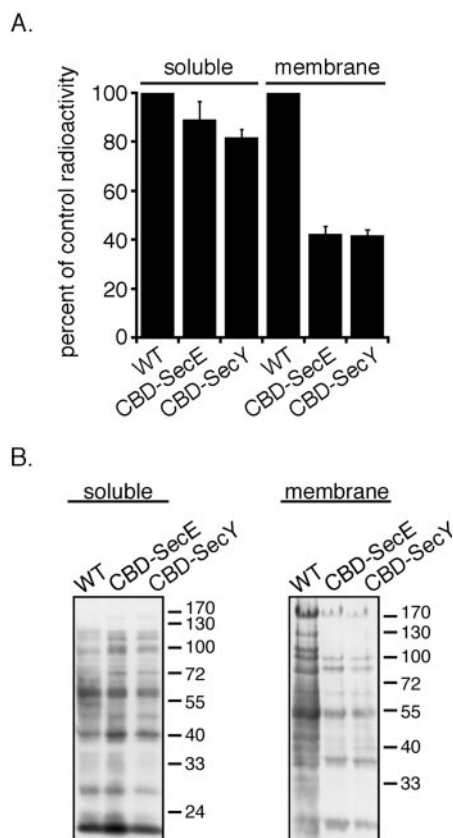


FIG. 3. Nascent membrane protein biogenesis is affected in CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells. A, the radioactivity associated with the soluble and membrane protein fractions of metabolically [35 S]cysteine/methionine radiolabeled background (WT) and CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells, grown to identical densities, was assessed by β -scintillation counting (A) or SDS-PAGE and fluorography (B). A, the radioactivity present in 5- μ l samples was measured. The values shown, expressed as percentages, represent the average of three experiments \pm S.D., where the radioactivity associated with the soluble and membrane protein fractions of the background cells is considered as 100%. B, fluorogram of radiolabeled soluble and membrane proteins from background (WT) and CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells, taken from a representative experiment. The positions of molecular weight markers are shown on the right.

cells, relative to the background. Striking differences between background and CBD-SecE- or CBD-SecY-expressing cells were, however, noted when membrane protein biogenesis was considered. The presence of either chimera led to decreases of close to 60% in the amount of newly synthesized membrane proteins over the period of radiolabeling. These findings, based on precipitation and scintillation counting of the radiolabeled protein content of each cell fraction, were qualitatively confirmed by SDS-PAGE and fluorography (Fig. 3B).

Decreased Ribosome Binding Modulates Protein Synthesis Rates—Given its profound effect on membrane protein biogenesis, the prolonged effect of decreased ribosome binding on cellular protein biosynthesis was next considered. In these experiments, background and CBD-SecE- and CBD-SecY-expressing cells were transferred to cysteine/methionine-free minimal medium for 90 min and then metabolically labeled with [35 S]cysteine/methionine for 5 h, during which time aliquots were taken at various intervals and examined in terms of the level of radiolabel incorporated into nascent polypeptides. This length of time was selected because it approximately corresponds to the generation time of *H. volcanii* cells (34, 40) and as such presents a reliable profile of protein synthesis in this species. In agreement with the earlier radiolabeling exper-

iments (Fig. 2), analysis of radiolabel incorporation 60 min after the onset of the labeling period revealed that significantly more radiolabeled nascent polypeptides had been synthesized in the background cells as compared with those *H. volcanii* cells expressing either CBD-SecE or CBD-SecY (Fig. 4A). With time, the rate of appearance of radiolabeled nascent polypeptides leveled off in the background strain, reaching a plateau after \sim 3.5 h. In contrast, after an initial lag phase lasting over an hour, the appearance of radiolabel-incorporating polypeptides increased at a constant rate in the CBD-SecE- and CBD-SecY-expressing cells throughout the remainder of the 5 h window of radiolabeling. Indeed, during this latter period, the rates of radiolabel incorporation in the CBD-SecE- and CBD-SecY-expressing cells (between 1.5 and 5 h) revealed similar slopes as the background strain, during the period of maximal protein synthesis (between 1 and 3.5 h) (1.0, 1.1, and 1.4, respectively). Analysis of the membrane protein contents of equivalent aliquots of the background, CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells by SDS-PAGE and fluorography revealed that each contained comparable amounts of *de novo* synthesized protein toward the end of the period of radiolabeling (Fig. 4B). Differences in profiles of newly synthesized membrane protein profiles in the background and transformed cells were, however, evident. Such differences could reflect an adaptation or other compensatory mechanism by the CBD-SecE- or CBD-SecY-expressing cells for the reducing ribosomal binding that occurs in these strains. Nonetheless, the appearance of comparable amounts of membrane proteins suggest that the transformed cells somehow managed to overcome any handicap associated with decreased ribosome binding due to the presence of CBD-SecE or CBD-SecY. This is supported by the similar growth rates of the background and transformed *H. volcanii* cells (Fig. 4C).

Finally, to determine whether the presence of CBD-SecE or CBD-SecY and the subsequently reduced level of ribosomal membrane binding also resulted in differential rates of protein degradation in the transformed *H. volcanii* cells relative to background strain, all three cell types were subjected to a 60-min [35 S]cysteine/methionine radiolabeling pulse followed by a 3-h chase with an excess of unlabeled cysteine and methionine. Under these conditions, analysis of the total level of polypeptide-incorporated radiolabel detected in aliquots sampled at various intervals during the chase period failed to reveal any significant differences in the rates of protein degradation (not shown). To discount for the possibility that a count of total radioactive incorporation would not accurately reflect incomplete protein degradation that had occurred during the course of the assay, the aliquots collected throughout the chase period were examined by SDS-PAGE and fluorography. In agreement with the scintillation counting results, no differences in protein pattern were detected among the background or CBD-SecE- or CBD-SecY-expressing cells (not shown). Finally, the possibility that the transformed cells degraded the CBD-SecE or CBD-SecY chimeras during the analyzed period of *de novo* protein synthesis could be discounted by the results of earlier studies that showed these proteins to be stably expressed in the membranes of transformed archaeal cells (41).

DISCUSSION

In the present communication, it was reported that the presence of CBD-SecE or CBD-SecY in *H. volcanii* cells led to substantial reduction in the number of ribosomes bound to the membrane, resulting in modulation of *de novo* membrane protein insertion. The finding that hindered ribosomal binding to the translocon selectively diminishes membrane protein biogenesis in *H. volcanii* provides the first evidence for the general co-translational insertion of membrane proteins in Archaea. To

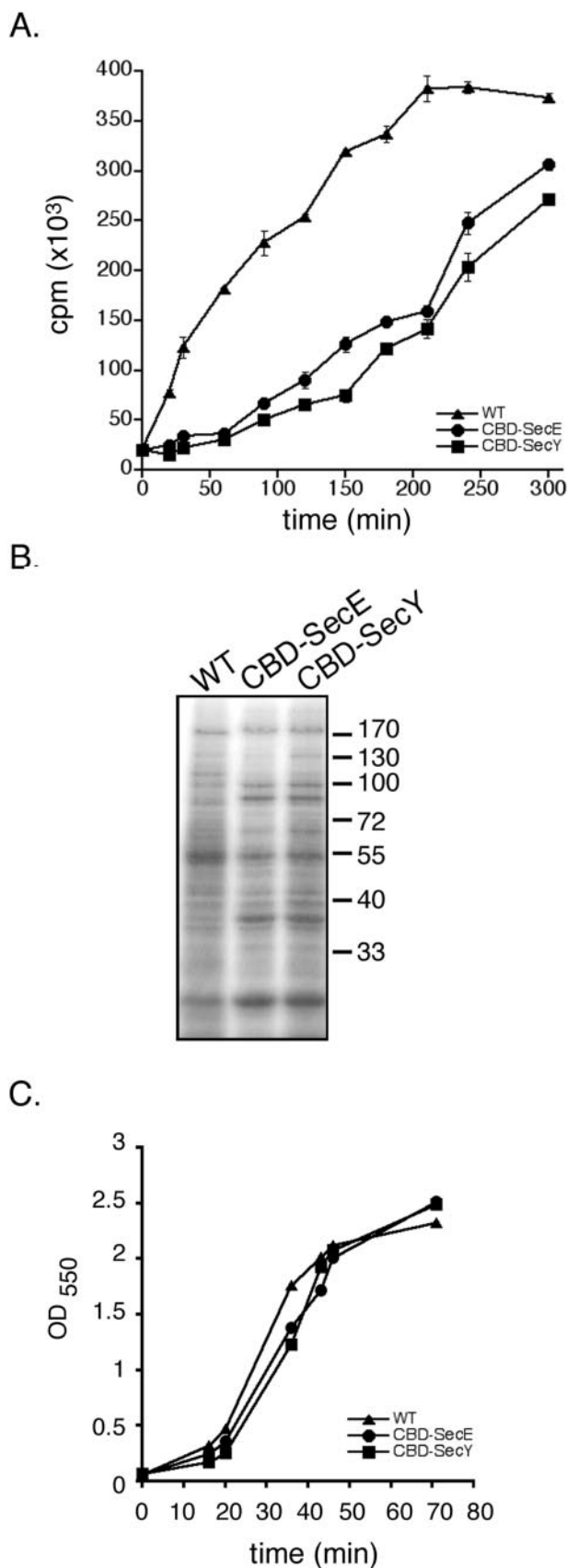


FIG. 4. Kinetic profiles of *de novo* protein synthesis in the different *H. volcanii* cells. **A**, background (WT) and CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells were grown to $A_{550} = 1.5$ at which time they were transferred to minimal medium lacking both cysteine and methionine. 90 min later, [³⁵S]cysteine/methionine radiolabeling mixture (14 μ Ci/ml) was added and aliquots removed at various intervals. The protein content of each aliquot was precipitated onto

date, a co-translational mode of membrane insertion in Archaea has been reported solely in the case of Bop (16–18), yet it remains unclear whether this reflects the use of a dedicated translocation machinery (19). The general use of a co-translational translocation system in Archaea is not, however, entirely unexpected, given the similarities between SRP in Archaea and Eukarya (20, 44), as well as the similar proportion of membrane-bound ribosomes in the two domains (Fig. 1A) (34, 35). In Eukarya, SRP is known to couple protein synthesis to protein translocation at the ER membrane (2), whereas membrane protein insertion involves translocon-bound ribosomes (45). Still, examples of post-translational protein translocation in Archaea have been reported (15). Thus, it is tempting to speculate that archaeal protein translocation involves post-translational secretion and co-translational membrane protein insertion. In Archaea, substantial numbers of both membrane and secreted proteins exist. A recent bioinformatics-based analysis predicted that in the ten complete archaeal genomes considered, ~10–25% of the predicted open reading frames in these species encode for exported proteins, with ~10–25% of these being secreted (46). In the case of *Halobacterium* sp. NRC-1, the only halophilic archaea for which complete genome sequence information has been published (47), 459 of the proposed 2630 open reading frames in this species are thought to correspond to exported proteins, with 68 of these being secreted and the rest being membrane-associated.

The ability of the transformed cells to eventually attain background strain rates of *de novo* protein synthesis following an initial lag period, as well as to achieve cell growth and steady-state membrane protein levels as in the background strain, reveals that the chimera-expressing cells were able to overcome the effects of reduced native translocon numbers and the resulting challenge to membrane protein insertion. Several explanations could account for this phenomenon. It is possible that faced with a decreased potential for ribosomes to bind to the membrane and hence participate in co-translational membrane protein insertion, *H. volcanii* cells could shift to a fully post-translational translocation mode. This shift in translocation strategy could explain the observed lag in *de novo* protein synthesis (Fig. 4A) and differential membrane protein profile (Figs. 3B and 4B) observed in the chimera-expressing cells. The changed membrane protein profile of the transformed cells, relative to the background strain, could reflect the biosynthesis of components involved in post-translational translocation or other proteins in response to this compensatory action. Indeed, post-translational secretion and membrane-protein insertion have been reported to occur in *H. volcanii* (15, 19).

Alternatively, CBD-SecE- and CBD-SecY-expressing cells could compensate for the lower number of bound ribosomes by retaining membrane-bound ribosomes at translocation-competent translocons following the termination of protein synthesis, as proposed by the Nichitta group based on their studies on ribosome detachment from the ER membrane (48–50) and mRNA partitioning in mammalian cells (36). Maintained ribosome binding at functional Sec sites would eliminate the putative delay associated with the seeking out of native translocons by membrane protein-synthesizing ribosomes in the CBD-

cellulose filters and subjected to scintillation counting. Each point represents the average of two experiments \pm S.D. **B**, fluorogram of radiolabeled membrane proteins from background (WT) and CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells after 5 h of radiolabeling. The results of a representative experiment are shown. The positions of molecular weight markers are shown on the right. **C**, cell growth of background (WT) and CBD-SecE- and CBD-SecY-expressing *H. volcanii* cells was measured at A_{550} . Each time point represents the average of three experiments, with a S.D. of <5%.

SecE- or CBD-SecY-expressing cells at the onset of each round of membrane protein biogenesis. By overcoming this kinetic obstacle to membrane protein insertion, the transformed cells could attain protein synthesis rates much higher than would be possible were each membrane protein-translating ribosome required to sample the entire translocon population before finding a functional site in these cells. Support for this concept comes with profile in protein synthesis presented in Fig. 4A, where a lag in protein synthesis in the chimera-expressing cells is seen prior to the realization of background strain rates. Because membrane-bound ribosomes, as reflected in Fig. 2C, were released during the pre-radiolabeling starvation phase, membrane protein-translating ribosomes must bind translocons *de novo* at the onset of the radiolabeling period. The lag in protein synthesis and delayed ribosome binding (Fig. 4B) observed in the CBD-SecE- or CBD-SecY-expressing cells could reflect the search by these membrane protein-translating ribosomes for functional translocons. The subsequent retention of ribosomes bound at translocons following the completion of membrane protein insertion is supported by the observation that puromycin treatment was unable to release membrane-bound *H. volcanii* ribosomes, implying that termination of protein synthesis in itself is insufficient for ribosomal release. This observation is in agreement with recent *in vivo* studies in a mammalian system, where 80 S ribosomes remained translocon-bound following puromycin-induced cessation of protein synthesis in human Jurkat T cells (49) and is also reminiscent of earlier *in vitro* studies in which exposure of rough microsomes to puromycin at physiological salt concentrations failed to release translocon-bound ribosomes (28). Further support for ribosomal detachment being a regulated process in *H. volcanii* came with the findings that the number of membrane-bound ribosomes depends on the physiological status of the cell (*i.e.* growth stage and nutrient levels) and that substantial amounts of both ribosomal subunits remain membrane-bound even following low salt washes of membranes isolated from puromycin-treated cells, despite the high salt requirement of *in vitro* *H. volcanii* ribosomal binding (32).

With the *in vivo* approaches described in this report, *in vitro* recreation of the ribosomal membrane-binding event (32), and recent advances made in solving the structures of the membrane-binding haloarchaeal 50 S ribosomal subunit (10) and of the archaeal translocon (11), additional insight into the archaeal ribosome-translocon interactions will be possible. Results of such studies will be relevant for understanding ribosomal function and membrane protein biogenesis not only in Archaea, but also across evolution.

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