Evidence for Post-translational Membrane Insertion of the Integral Membrane Protein Bacterioopsin Expressed in the Heterologous Halophilic Archaeon *Haloferax volcanii*

Ron Ortenberg and Moshe Mevarech‡

From the Department of Molecular Microbiology and Biotechnology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel

The gene coding for the integral membrane protein bacterioopsin (Bop), that is composed of seven transmembrane helices, was expressed in the halophilic archaeon *Haloferax volcanii* as a fusion protein with the halobacterial enzyme dihydrofolate reductase and with the cellulose binding domain of *Clostridium thermocellum* cellulosome. In each case, bacterioopsin was present both in the membrane and in the cytoplasmic fractions. Pulse-chase labeling experiments showed that the fusion protein in the cytoplasmic fraction is the precursor of the membrane-bound species. Bacterioopsin mutants that lack the seventh helix (BopΔ7) were found to accumulate only in the cytoplasmic fraction, whereas bacterioopsin mutants that lack either helices four and five (BopΔ4–5), or helices one and two (BopΔ1–2), were found in the cytoplasmic as well as in the membrane fractions. The seventh helix, when expressed alone, could target in trans the insertion of a separately expressed bacterioopsin mutant protein that has only the first six helices. These results support a model in which bacterioopsin is produced in *H. volcanii* as a soluble protein and in which its insertion into the membrane occurs post-translationally. According to this model, membrane insertion is directed by the seventh helix.

SRP is not essential for the survival of the yeast cell (10). Despite the accumulating knowledge regarding the mechanism of insertion of integral membrane proteins in eukaryotes and bacteria, virtually nothing is known about this process in archaea. Recently, several genes that code for putative components of the secretion/translocation machinery in archaea were identified by searching the sequences of complete archaeal genomes for genes homologous to those known to be involved in secretion (11). Unfortunately, these studies are still incomplete, and there are, to date, no genetic and biochemical data to confirm them.

The biogenesis of bacteriorhodopsin is an excellent model system to analyze the assembly of polytopic membrane proteins. Bacterioopsin (Bop) is an integral membrane protein composed of seven transmembrane helices and is produced by the halophilic archaeon *Halobacterium salinarum* (12) and by some other halobacterial species (13). The complex of Bop with the chromophore, all-trans-retinal, forms bacteriorhopdopsin, which is organized in clusters that are termed "purple membranes." Bacteriorhodopsin functions as a light-driven proton pump that upon illumination generates a proton gradient that is used to produce ATP. The photoreaction cycle of bacteriorhodopsin in the purple membrane has been extensively characterized (14), and the structure of bacteriorhodopsin in the purple membrane has been determined to high resolution (15, 16). In addition to bacteriorhodopsin, *H. salinarum* produces three retinal binding proteins: sensory rhodopsin I and II (SopI and SopII, respectively), which serve as light-sensing molecules (17), and halorhodopsin (Hop) (18), which serves as a Cl⁻ pump (19). Although SopI, SopII, and Hop have the same topology as bacteriorhodopsin, amino acid sequence relatedness between the four species is limited.

Despite the considerable knowledge of the biophysical properties of bacteriorhodopsin, relatively little is known about the biogenesis of the purple membrane. It has been shown that halobacterial 7 S RNA sediments with the ribosomal fraction that translates the bacterioopsin mRNA (20). Since 7 S RNA is an integral part of the SRP complex, it was suggested that Bop is inserted into the membrane co-translationally. In agreement with this hypothesis, Heather and Krebs (21) have recently shown that the N terminus of Bop is inserted into the membrane of *H. salinarum* co-translationally.

This report describes the heterologous expression of the *H. salinarum* bop gene in the halophilic archaeon *Haloferax volcanii*, which lacks the genetic capacity to produce bacterioopsin. We present evidence that in *H. volcanii* bacterioopsin is incorporated into the membrane post-translationally and that the seventh transmembrane helix of the protein plays a critical role in this process.
**Experimental Procedures**

**Bacterial Strains and Growth Media**

*H. volcanii* white mutant (SX) (kindly obtained from Dr. Robert Charlebois, University of Ottawa, Ottawa, Canada). *H. volcanii* methionine/cysteine auxotrophic strain (WR341). *H. salinarum* S9-Bop constitutively expressing strain, and *H. salinarum* L33-Bop deficient strain were used in the present work. Rich medium (H) and agar plates were prepared as described previously (22) with one modification (50 mM Tris-HCl, pH 7.2, was used as the buffer). For selection of transformants, the medium was supplemented with 1 μg/ml novobiocin (Noc) (Sigma). Trimethoprim (Sigma) at a final concentration of 2 μg/ml and all-trans-retinal (Sigma) at a final concentration of 10 μM were added to the medium when required. *Escherichia coli* strain TG1 was grown in Luria-Bertani (LB) medium. When needed, ampicillin (Sigma) was added to the medium to a final concentration of 100 μg/ml.

**Molecular Genetic Methods**

Restriction endonuclease digestion, agarose gel electrophoresis, and molecular cloning were performed according to standard procedures described by Maniatis *et al.* (23). PCR amplification was carried out with a 200 mM concentration of each primer, a 200 μM concentration of each dNTP, 10–50 ng of plasmid template DNA, and 2.5 units of Taq DNA polymerase (TaKaRa Shuzo Co., Otsu, Shiga, Japan) in a final volume of 100 μl. Thirty amplification cycles were performed (30 s at 94°C (denaturation step), 30 s at 55°C (primer annealing), and 30 s at 72°C (polymerization step)). The PCR products were purified by the High Pure™ PCR Product Purification Kit (Roche Molecular Biochemicals) as recommended by the supplier. Transformation of halobacteria was carried out as described previously (24). *E. coli* TG1 was transformed according to the CaCl2 protocol (23).

**Preparation of Membrane and Cytoplasmic Fractions**

**Preparation of Membrane and Cytoplasmic Fractions at Low Salt Concentration**—Halobacterial strains were grown for 3 days to early stationary phase. The cell suspension (50 ml) was centrifuged at 3000 × g for 10 min. The cells in the pellet were lysed by resuspension in 5 ml of 0.5 M NaCl, 0.5 mM MgAc, and 0.5 mM Mops (pH 7.2). The membrane and cytoplasmic fractions were separated by centrifugation at 40,000 × g (18,500 rpm) for 1 h in a Sorvall SS-34 rotor. The supernatant was recentrifuged twice and analyzed as a cytoplasmic fraction in further experiments. The membrane pellet was resuspended in 2 ml of distilled water, recentrifuged twice, and then resuspended in 0.5 ml of distilled water for further analysis as a membrane fraction. When purification of the cell-free extract was required, 50 μl of a 10% (w/v) cellulose suspension (Sigma) was added to 1 ml of the cytoplasmic fraction, and the mixture was incubated for 30 min at room temperature while continuously inverting the tube. After incubation, the cellulose suspension was washed twice with 0.5% (w/v) Tween 20 solution. Recovery of the cellulose-bound proteins was performed by boiling the cellulose pellet in 50 μl of the SDS gel loading buffer.

**Preparation of Membrane and Cytoplasmic Fractions at High Salt Concentration**—Halobacterial strains were grown for 3 days to early stationary phase. The cell suspension (50 ml) was centrifuged at 3000 × g for 10 min. The pellet was resuspended in 5 ml of the high salt solution (3 M KCl, 100 mM MgAc, 10 mM Hpes) in the presence of DNase I (Sigma) at a final concentration of 25 μg/ml and sonicated. The membrane and cytoplasmic fractions were separated by ultracentrifugation at 100,000 × g (35,000 rpm) for 1 h in a SW 50.1Ti rotor (Beckman). The supernatant was recentrifuged and served as the cytoplasmic fraction in further experiments. The membrane pellet was resuspended in 2 ml of distilled water, recentrifuged, and resuspended in 0.5 ml of distilled water for further analysis as the membrane fraction. When purification of the cellulose-binding proteins from the cytoplasmic fraction was required, 50 μl of a 10% (w/v) cellulose suspension (Sigma) was added to 1 ml of the cytoplasmic fraction, and the mixture was incubated for 30 min at room temperature while continuously inverting the tube. After incubation, the cellulose suspensions were washed twice with 0.5% (w/v) Tween 20 solution. Recovery of the cellulose-bound proteins was performed by boiling the cellulose pellet in 50 μl of SDS gel loading buffer.

**Western Blot Analysis**

Samples of protein extracts were electrophoresed in standard SDS-polyacrylamide gels (10% (w/v) or 12% (w/v)). The gels were electroblotted onto nitrocellulose filters and treated with antibodies against hDHFR or cellulose binding domain (CBD), followed by exposure with goat-anti-rabbit IgG conjugated to peroxidase. The bound antibody was detected by the ECL kit (Amersham Pharmacia Biotech).

**Flotation Gradient Analysis of the Membrane Fraction**

Flotation gradient analysis was performed as described (25) with slight modifications. The membrane fraction from *H. volcanii* cells were prepared at low salt concentration as described above with the following modification. The final membrane pellet was resuspended in 0.5 ml of buffer I (50 mM NaCl, 1 mM EDTA pH 8, 100 mM Tris-HCl, pH 8, 7.5% (w/v) sucrose). 40 μl of the membrane suspension was mixed with 400 μl of buffer II (50 mM NaCl, 1 mM EDTA pH 8, 100 mM Tris-HCl, pH 8, 5% (w/v) sucrose) and transferred to the bottom of an ultracentrifuge tube for the TLS 55 swing-out rotor (Beckman). The sample was overlaid with 880 μl of buffer III (50 mM NaCl, 100 mM Tris-HCl, pH 8.0, 52% (w/v) sucrose) and 270 μl of buffer I. After ultracentrifugation for 4 h at 250,000 × g (54,000 rpm), 200 μl were removed from the top of the gradient, and the flotation gradient was collected in three fractions (400, 300, and 490 μl), designated as the top, middle, and bottom fractions. The fractions were trichloroacetic acid-precipitated and analyzed by Western blot. The optical densities of the ECL bands were quantified using TINA software.

**Spectroscopy Analysis**

Membrane fractions were prepared from the *H. volcanii* SX/S8CB and SX strains and from the *H. salinarum* S9 and L33 strains prepared at low salt concentration as described above. The optical absorbance spectra (450–700 nm) of total membrane proteins prepared from the SX/S8CB and S9 strains was obtained employing an Ultraspec III spectrophotometer (Amersham Pharmacia Biotech), using the membrane fractions prepared from the SX and L33 strains as the corresponding references.

**Pulse and Pulse-Chase Experiments**

A 200-μl culture of the *H. volcanii* methionine auxotrophic strain WR341 expressing the Bop-CBD fusion protein was grown in rich medium to an A590 of 0.5, centrifuged, and resuspended in 10 ml of minimal medium containing 4 mM NaCl, 5 mM KCl, 150 mM MgSO4, Tris, 50 mM, pH 7.2, 0.05% (w/v) CaCl2, 0.67% (w/v) yeast nitrogen base (Difco), 0.05% (w/v) glycerol, 0.5% (w/v) sodium succinate and a mixture containing all of the standard amino acids except methionine and cysteine at a final concentration of 40 μg/ml each. The culture was shaken for 30 min at 37°C, and then 300 μCi of [35S]methionine (7000 Ci/mmol) was added. Incubation was continued, and aliquots of 1 ml were removed every 20 min and centrifuged immediately at 3000 × g for 3 min.

In pulse-chase experiments, after an incubation of 1.5 h with the labeled methionine, 1 ml was removed (time 0), and unlabeled l-methionine was added to a final concentration of 40 μg/ml. Samples of 1 ml were taken every 20 min, aliquots were immediately added to 3000 × g for 3 min. The cells were lysed by resuspension in 2 ml of H2O in the presence of 25 μg/ml DNase I. The membrane and cytoplasmic fractions were separated by centrifugation at 40,000 × g for 1 h in a Sorvall SS-34 rotor. The supernatant was analyzed as the cytoplasmic fraction. The membrane pellet was resuspended in 100 μl of 10% (v/v) Tween 20 solution, and 200 μl of H2O was added. To each of the membrane and cytoplasmic fractions, 50 μl of a 10% (w/v) cellulose suspension (Sigma) was added. After incubation for 30 min at room temperature, the cellulose suspensions were washed twice with 0.5% (v/v) Tween 20 solution. Recovery of the Bop-CBD was performed by boiling of the cellulose pellet in 40 μl of SDS gel loading buffer. Samples of 20 μl were electrophoresed on SDS-polyacrylamide gel (10%, w/v). The gel was dried and exposed to a phospho-image screen (Fuji) for 4–12 h. The spectra were scanned in a phospho-image reader (BAS 1000, Fuji) and analyzed using TINA software.

**Plasmid Construction**

Recombinant plasmids for expression of the different *bop* gene fusions were constructed as follows (see Fig. 1). The gene coding for the *H. salinarum* bacterioopsin (*bop*) was PCR-amplified from the plasmid pEF1100 (26) while generating an Nco I site at the 5′-end and a BigI site at the 3′-end. The 5′-end of the fragment was fused to the synthetic *H. volcanii* constitutive promoter PrtR16 (27). The 3′-end of the *bop* gene was fused in-frame to the cytoplasmic fraction. The 5′-end was generated with the PCR-primer gene coding for hDHFR (28) via a linker consisting of the nucleotide sequence coding for the first 33 codons of the *H. salinarum* ferredoxin gene (29). The entire construct was cloned into the pWt-Nov shuttle vector, which contains *E. coli* and *H. volcanii* replication origins and an ampicillin resistance gene for
selection of transformants in *E. coli* and a novobiocin resistance gene (30) for selection of transformants in *H. volcanii*. The final construct was named pNBLD38. The *cbd* gene (coding for *Clostridium thermocel-
lum* cellulose cellulose binding domain) was amplified by PCR using primers that introduce the *Bgl*I and *Xba*I restriction sites at the 5’- and 3’-ends of the gene, respectively. The part of the *bop-hdrA* gene containing the linker-hDHFR fusion in pNBLD38 plasmid was replaced with the *cbd* gene using the *Bgl*I and *Xba*I restriction sites to yield the plasmid pNB38CBD.

Plasmid pHE1, which contains the gene coding for hDHFR, fused directly to the strong promoter PrR16 via the same linker as used in pNBLD38 and was used as a control plasmid.

**Construction of Bop Deletion Mutants**

Five Bop mutants containing deletions within the protein were created using the *bop-hdrA* construction as follows (see Fig. 5).

**pNBLD46**—The *bop* gene was deleted for the domain encoding the seventh transmembrane helix and the intracellular C terminus (the polypeptide fragment corresponding to amino acids from position 212 to 262); the linker-hDHFR fragment was fused to the extracellular loop flanking the sixth helix through a *Bgl*I site introduced by PCR at position 633 of the gene. The pNB46 plasmid carries the same deletion in the *bop* gene as pNBLD46; however, the region encoding the linker and the hDHFR has been removed and a stop codon introduced at position 633 of the gene.

**pNBLD48**—The *bop* gene was deleted for the domains encoding the last two transmembrane helices (the polypeptide fragment correspond-
ing to amino acids from position 179 to 262), and the linker-hDHFR fragment was fused to the intracellular loop flanking the fifth transmembrane helix through a *Bgl*I site introduced by PCR at position 536 of the gene.

**pNBLD54**—The *bop* gene was deleted for the domains encoding transmembrane helices four and five (the polypeptide fragment corre-
sponding to amino acids from position 121 to 174) and was constructed by PCR by introducing *Bgl*I and *Bam*HI sites after the third helix at position 361 of the gene and before the sixth helix at position 521 of the gene, respectively, following digestion with these two enzymes and self-ligation.

**pNBLD56**—The *bop* gene was deleted for the domains encoding the first two transmembrane helices (the polypeptide fragment correspond-
ing to amino acids from position 22 to 85) and was constructed by removal of the region between the *Aar*II restriction site at position 56 of the gene and a new *Aar*II site introduced by PCR before the third helix at position 257 of the gene.

**pNBLD58**—The *bop* gene is deleted for the domains encoding transmembrane helices one to six (the polypeptide fragment corresponding to amino acids from position 22 to 212) and was constructed by removal of the region between the *Aar*II restriction site at position 56 of the gene and a new *Aar*II site introduced by PCR before the seventh helix at position 633 of the gene.

**RESULTS**

**Construction of Bop Expression System in *H. volcanii***—Plasmid vectors constructed in this work for the expression of the *H. salinarum* *bop* gene in *H. volcanii* are shown in Fig. 1. The fused hDHFR gene provides a positive selection for the Bop-
hDHFR chimera in *H. volcanii* as hDHFR confers resistance to the anti-folate drug trimethoprim. It also enables the detection of the fused protein by antibodies raised against hDHFR. The pNB38CBD plasmid contains a fusion of the *bop* gene to the gene coding for the CBD of the *C. thermocellum* cellulose (31). The fusion enables an easy affinity purification of the Bop-CBD chimera by binding to cellulose. The plasmid pHE1, which contains the hDHFR gene fused directly to the strong promoter PrR16, was constructed to serve as a control plasmid.

All three recombinant plasmids contain replication origins for propagation in *E. coli* and *H. volcanii*, an ampicillin resistance gene for selection of *E. coli* transformants, and an apramycin resistance gene (30) for selection of *H. volcanii* transformants.

The recombinant plasmids pNBLD38, pNB38CBD, and pHE1 were introduced into *H. volcanii* SX (white mutant), and transformants were selected for on novobiocin plates. Cells bearing pNBLD38 (SX/38) were found to be resistant to 2 μg/ml trimethoprim, as were cells containing pHE1 (SX/1), whereas growth of untransformed cells and cells containing pNB38CBD (SX/38CBD) was completely inhibited by 2 μg/ml trimethoprim (MIC<sub>Top</sub> for SX cells is 0.25 μg/ml).

The ability of transformants containing pNBLD38 and pNB38CBD to produce bacteriorhodopsin was examined after growth in the presence of 10 μM retinal. Cultures of both SX/38 and SX/38CBD strains became purple (data not shown), whereas cultures of untransformed cells as well as of SX/1 cells grown in the presence of the same concentration of retinal remained white.

**Analysis of Bop Expression in *H. volcanii***—To analyze the synthesis and cellular localization of Bop in *H. volcanii*, membrane and cytoplasmic fractions from transformants containing pNB38CBD and from control cells lacking the plasmid were prepared at the low and high salt concentration conditions (as described under “Experimental Procedures”). Fig. 2 shows that the Bop-CBD fusion protein occurs both in the cytoplasmic and in the membrane fractions. Membrane and cytoplasmic fractions were prepared from equivalent amounts of cells and analyzed by SDS-PAGE (Fig. 2A) and by Western blot analysis using antibodies against CBD (Fig. 2B). It was found that the amounts of the Bop-CBD fusion protein obtained in the cyto-
plasmic and membrane fractions were similar in the samples prepared either at low or high salt concentration and separated by centrifugation at 40,000 or 100,000 × g, respectively. Similar results were obtained when cytoplasmic and membrane fractions, prepared from cells expressing the Bop-hDHFR chi-
mera, were analyzed by Western analysis using antibodies against hDHFR (data not shown).

Flotation gradient analysis was carried out to verify that the Bop-CBD protein found in the membrane fraction was inserted into the membrane. Most of the Bop-CBD fusion protein was detected in the top (about 68%) and in the middle (about 23%) layers of the step gradient (Fig. 3). In these experiments, pro-
teins will normally float from the loading zone at the bottom of the gradient to the top and middle fractions only if they are integrated into the membrane. The results obtained here suggest that the Bop-CBD fusion protein is stably integrated into the membrane.

In order to address the question of whether the membrane-bound Bop-CBD fusion protein is properly folded, membrane fractions were prepared from cells of the SX/38CBD strain grown in the presence of retinal and from the *H. salinarum* S9 (Bop constitutively expressing strain), as described under “Ex-

**FIG. 1.** Schematic illustration of the pNBLD38, pNB38CBD, and pHE1 plasmids. The three recombinant plasmids were constructed as described under “Experimental Procedures.” The positions of the restriction sites used for cloning are indicated.
In Vivo Membrane Insertion of Bacterioopsin

**Characterization of Bop Deletion Mutants—**Bop deletion mutants were created to help identification of the region(s) within the protein involved in membrane targeting. The membrane preparations were diluted to the same optical density at 568 nm (the absorption maximum of bacteriorhodopsin (12)). Equivalent samples from both suspensions were electrophoresed on a 12% (w/v) SDS-polyacrylamide gel, stained by Coomassie Brilliant Blue, and photographed, and the absorption profiles of the protein bands were determined by TINA software. The absorption profiles of the corresponding bands of native Bop, prepared from the *H. salinarum* S9 strain, and Bop-CBD, prepared from the *H. volcanii* SX/38CBD, were very similar, implying that the Bop-CBD chimeric protein is correctly folded in the membrane (data not shown).

**Kinetics of Bop Biogenesis in *H. volcanii*—**Pulse-chase experiments were performed to follow the kinetics of Bop biogenesis in *H. volcanii*. Plasmid pNB38CBD containing the bop-cbd fusion was transformed into *H. volcanii* WR341 (Met^+^) strain to yield the WR341/38 strain. Pulse labeling was carried out by the addition of [35S]methionine to a mid-log phase culture of the WR341/38 strain, and samples were removed every 20 min as described under “Experimental Procedures.” Fig. 4A shows the amount of labeled Bop-CBD protein present in the cytoplasmic and membrane fractions prepared from these samples at different times. Labeled Bop-CBD fusion protein occurs first in the cytoplasmic fraction and only later can be detected in the membrane fraction. When the labeled cells were subjected to a large excess of nonlabeled methionine, the radioactivity in the Bop-CBD fusion protein in the cytoplasmic fraction decreased with time, whereas the amount of radioactivity in the membrane fraction increased (Fig. 4B). These two processes occurred at similar rates, indicating that the cytoplasmic form of the Bop-CBD fusion protein is a precursor of the membrane form of this protein.

**Fig. 2.** SDS-PAGE and Western analyses of Bop-CBD chimera expression in *H. volcanii*. Cytoplasmic and membrane fractions were prepared from cells that carry no plasmid (SX(A1)) or the pNB38CBD plasmid (SX/38CBD) employing low (A2) or high (A3) salt concentration conditions as described under “Experimental Procedures.” The cytoplasmic form of the Bop-CBD chimera was purified from the cytoplasmic fraction of SX/38CBD after binding to cellulose as described under “Experimental Procedures”; the same procedure was performed with the cytoplasmic fraction prepared from cells of the SX strain as a control. Samples of the membrane fractions (c) and of the Bop-CBD cytoplasmic form (c) prepared from an equivalent amount of cells were analyzed by SDS-PAGE 12% (w/v) (A) and by Western blot using antibodies against CBD (B). The molecular mass (kDa) markers are indicated.

**Fig. 3.** Flotation gradient analysis of the membrane fraction containing Bop-CBD chimera. 40 ml of the membrane suspension prepared from cells of the SX/38CBD strain were subjected to sucrose step gradient analysis as described under “Experimental Procedures.” The gradient was divided into three fractions, which were analyzed by Western blot using antibodies against CBD. The densities of the bands were quantified using TINA software.

The ability of the transformants to grow in the presence of trimethoprim was determined in liquid culture. All of the transformants were resistant to 2 μg/ml trimethoprim. None of the cultures bearing plasmids with a deletion in the *bop* gene produced a purple color when grown in the presence of all-trans-retinal.

Membrane and cytoplasmic fractions were prepared from cells harboring each of the above plasmids with different mutant *bop* genes and from cells that harbor pNBLD38 (containing the wild type *bop* gene) and analyzed by Western blot using antibodies against hDHFR. Fig. 5B shows that the fusion protein derived from BopΔ7 that lacks the polypeptide domain containing the seventh transmembrane helix together with the C-terminal cytoplasmic tail and the protein from BopΔ6–7 that lacks both the seventh and sixth transmembrane helices both occur only in the cytoplasmic fraction. In contrast, the wild type Bop and the fusion proteins derived from Bop mutants that lack the first two helices, BopΔ1–2, or the fourth and fifth helices, BopΔ4–5, occur in both the cytoplasmic and membrane fractions. In all cases, the cytoplasmic forms of the Bop fusion protein were unstable, and degradation products were detected as bands with lower molecular weight.

**The Role of the Seventh Helix of Bop in Membrane Insertion—**According to the results described above, the seventh transmembrane helix of Bop appears to play a critical role for Bop insertion into the membrane. To determine whether the seventh helix alone can drive the attachment of hDHFR to the membrane, plasmid pNBLD58 (BopΔ1–6), which codes for a fusion protein in which the seventh helix alone is fused to hDHFR, was constructed (Fig. 6). Attempts to transform this plasmid into the *H. volcanii* SX strain were unsuccessful despite the fact that transformants with the control plasmids pNBLD38 and pHE1 were obtained at usual frequencies (10^7 transformants/μg of plasmid DNA). Expression of the seventh helix by itself appears therefore to be toxic to the cells.

However, when pNBLD58 (BopΔ1–6) was introduced into cells that contain pNB46 (BopΔ7) and express BopΔ7 (Fig. 6), transformants resistant to both novobiocin and trimethoprim were readily obtained at normal frequencies. Membrane and cytoplasmic fractions were prepared from the SX/58 cells that carry both pNBLD58 and pNB46 plasmids and analyzed by
Western blot using antibodies against hDHFR. Fig. 6 shows the presence of a protein band corresponding in size to that of the seventh transmembrane helix fused to hDHFR in the cytoplasmic fraction. Significantly, in the membrane fraction a band was detected with the same antibodies with a much higher molecular mass (about 49 kDa), which corresponds to the molecular mass of the Bop-hDHFR chimera.

**DISCUSSION**

Rationale of the Experimental Approach—In the past, the elucidation of purple membrane biogenesis in *H. salinarum* was approached by the characterization of mutants deficient in purple membrane formation. This approach yielded mutants that were defective in *bop* gene expression but failed to produce mutants defective in the protein translocation machinery. In order to circumvent this problem, we attempted to transfer the genetic capacity to produce functional bacteriorhodopsin from *H. salinarum* to the halophilic archaeon *H. volcanii*. We supposed that if *H. volcanii* could be made to produce the purple membrane, it would have acquired all of the necessary genetic elements needed for purple membrane biogenesis. Since the *H. salinarum* *bop* promoter is not functional in *H. volcanii*, the *bop* structural gene was cloned into a halobacterial plasmid under a constitutive halobacterial promoter. Expression of *bop* in *H. volcanii* was not expected to confer a selectable phenotype, and to this end a gene fusion was created between the *H. salinarum* *bop* gene and the *H. volcanii* *hdrA* gene coding for the enzyme dihydrofolate reductase. The latter provides resistance of *H. volcanii* to the anti-folate inhibitor trimethoprim. To facilitate purification of Bop, the *bop* gene was fused to the nucleotide sequence encoding the cellulose binding domain of the *C. thermocellum* cellulose.

It was recently shown that a Bop-hDHFR fusion protein expressed in a strain of *H. salinarum* deleted for the *bop* gene was successfully incorporated into the membrane and that its molecular packing in the membrane closely resembled the ordered structure of the wild type bacteriorhodopsin in the purple membrane (32). Also, the activity of both parts of the bifunctional protein was demonstrated. Moreover, the hDHFR portion of the Bop-hDHFR chimera was detected only on the cytoplasmic side of the plasma membrane, confirming that the proper molecular orientation of the chimera in the membrane obtained. Similar results were obtained when Bop was expressed as a fusion protein with the green fluorescent protein of *Aequorea victoria* (33) and with aspartyl transcarbamylase (34). These observations suggest that expression studies of different Bop chimeric proteins could serve as a useful model for understanding bacterioopsin biogenesis.

*Bop Is Inserted into the H. volcanii Membrane Post-translationally*—In the present study, we show that expression of the genes coding for the Bop-hDHFR and Bop-CBD chimeras in *H. volcanii* results in a membrane-bound bacterioopsin. When the membrane fraction was subjected to flotation gradient analysis, most of the Bop-CBD protein was detected in the top fraction of the gradient, suggesting that the Bop-CBD chimera forms an integral part of the membrane. No significant differences were observed between the visible spectra of the Bop-CBD chimera prepared from *H. volcanii* and wild type Bop prepared from *H. salinarum* S9. Surprisingly, an appreciable amount of the Bop fusion proteins were present in the cytoplasmic fraction.

The existence of the cytoplasmic form of Bop could be due to two reasons: first, the cytoplasmic fraction might contain misfolded forms of the Bop chimera that are unable to integrate into the membrane; second, the cytoplasmic form of Bop might be the precursor of the membrane form. Pulse label experiments showed that the newly synthesized Bop-CBD appears initially in the cytoplasm and subsequently is found in the membrane. The slow rate of Bop incorporation into the membrane (Bop was detected in the membrane only at 20 min after administering the pulse) can be explained by the fact that even in optimal growth conditions *H. volcanii* is a slow grower (its doubling time is about 6 h), and in the conditions used in this study its growth was even slower.

Pulse-chase experiments revealed that the cytoplasmic species is converted to a membrane-attached form, which is pre-
sumably the precursor of the membrane species. Based on these observations, we propose that, when produced in *H. volcanii*, bacterioopsin is assembled in the membrane from a cytoplasmic precursor by a post-translational mechanism. This view is supported by studies using Bop deletion mutants. Thus, localization of the fusion protein in the membrane fraction required the presence of an intact seventh transmembrane segment of Bop, whereas neither the fourth and fifth nor the first and second transmembrane segments were essential for membrane integration. If Bop is inserted co-translationally, it is difficult to explain the fact that deletion of the seventh helix of Bop prevents the insertion of the mutant protein into the membrane.

The Last Helix of Bop Acts as a Membrane Targeting Signal—Attempts to transform *H. volcanii* with a plasmid coding for a fusion between the seventh (and last) transmembrane helix of Bop and DHFR (Bop<sup>D7</sup>) were unsuccessful, suggesting that expression of the seventh helix of Bop alone in *H. volcanii* is toxic for the cells. This helix is expected to form an amphipathic polypeptide with a highly hydrophobic nonpolar surface and a polar surface. Possibly, the toxicity of the Bop seventh helix might be similar to that observed when cells are exposed to natural or synthetic amphiphatic peptides that disrupt membranes (35, 36). On the other hand, when the Bop<sup>D1–6</sup> fusion protein was expressed in cells that express Bop<sup>D7</sup>, i.e. a polypeptide containing each of the first six transmembrane helices, the seventh helix was no longer toxic to the cells and could be detected in the membrane. The molecular mass of this membrane form is similar to the molecular mass of the complete Bop-hDHFR chimera. This latter result suggests that the seventh helix can interact with Bop<sup>D7</sup> to form a membrane complex that is stable even in the presence of SDS. The noncovalent, SDS stable, attachment between transmembrane α-helices has previously been described and may take place as an essential step in the assembly of integral membrane proteins (37, 38). Moreover, it has been reported that bacteriorhodopsin can be refolded to the native state *in vitro* from its proteolytic fragments (39) or from its polypeptides expressed in *E. coli* (40). These results point to the possibility that during the process of Bop folding some helix-helix interactions do not require formation of covalent linkages, a view that is compatible with previously published observations that show that co-expression of adjacent fragments of rhodopsin (41, 42).
In Vivo Membrane Insertion of Bacterioopsin

42), the lactose permease (43), the red blood cell anion exchanger protein (44, 45), and the β-barrel membrane protein OmpA (46) leads to in vivo assembly of a functional protein. We presume that the seventh transmembrane helix of Bop serves both as a membrane targeting signal and in facilitating helix-helix interactions with the other Bop helical domains and that it promotes the formation of the pretranslation form of Bop, which enables its insertion into the membrane.

The Nature of the Cytoplasmic Form of Bop—Bacterioopsin is an “inside-out” protein (47). The charged and polar groups of the bacterioopsin molecule tend to lie at the molecular interior, while the nonpolar surfaces are directed outward. What then is the nature of the soluble cytoplasmic form of bacterioopsin? There are several examples of specific molecular chaperons that are involved in the folding or assembly of proteins destined to be integral membrane proteins. In Drosophila, the biogenesis of rhodopsin depends on the presence of a photoreceptor cell-specific cyclophilin, NinaA, which functions as a chaperone. In ninaA mutants, rhodopsin is retained within the endoplasmic reticulum, and its levels are reduced by more than 100-fold (48). The membrane insertion of sensory rhodopsin I (SopI) found in H. salinarum is dependent on a chaperone-like function of its signal transducer, HtrI, which facilitates membrane insertion and protein folding of SopI (49). It was also shown that the chaperonin GroEL can promote post-translational membrane insertion of SopI (49). It was also shown that the chaperonin GroEL can promote post-translational membrane insertion and protein folding of SopI (49). It was also shown that the chaperonin GroEL can promote post-translational membrane insertion and protein folding of SopI (49). It was also shown that the chaperonin GroEL can promote post-translational membrane insertion and protein folding of SopI (49).

In contrast, H. volcanii does not naturally encode the bop gene and the highly efficient co-translational biochemical machinery responsible for its membrane insertion. Membrane insertion of Bop in H. volcanii is therefore, not unexpectedly, a slower post-translational process. Nevertheless, this model might apply for insertion of other halobacterial seven-transmembrane helix-containing proteins that do not have the Bop signal peptide or a specific chaperon system that promotes their membrane insertion. Also, the heterologous H. volcanii system should facilitate a more complete characterization of the genetic and biochemical components of the Bop membrane insertion machinery present in H. salinarum.

Acknowledgments—We thank Dr. Eitan Bibi and Anat Herskovits for suggesting and helping in the flotation gradient analysis, Dr. Gerald Cohen and Dr. David Gutnick for critical reading of the manuscript, Dr. Rafi Lamed for providing the gene coding for the cellulose binding domain of C. thermocellum cellulose, and Yehudit Navon for technical assistance.

REFERENCES

1. Bibi, E. (1998) Trends Biochem. Sci. 23, 51–55
2. Zelanya, A., Seluanov, A., Cooper, A., and Bibi, E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6025–6029
3. Valenti, Q. A., Scotti, P. A., High, S., de Gier, J. W., von Heijne, G., Lenzten, G., Wintemeyer, W., Ouédraogo, B., and Luirink, J. (1998) EMBO J. 17, 2504–2512
4. Ulbrandt, N. D., Newitt, J. A., and Bernstein, H. D. (1992) Cell 88, 187–196
5. de Gier, J. W., Scotti, P. A., Saaaf, A., Valenti, Q. A., Kuhn, A., Luirink, J., and von Heijne, G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 14646–14651
6. Brodsky, J. L. (1998) Int. Rev. Cytol. 178, 277–328
7. Rapoport, T. A., Jungnickel, B., and Kutay, U. (1996) Annu. Rev. Biochem. 65, 271–303
8. Roth, C., and Lehle, L. (1998) Eur. J. Biochem. 252, 16–24
9. Ng, D. T., Brown, J. D., and Walter, P. (1996) J. Cell Biol. 134, 269–278
10. Ogg, S. C., Poritz, M. A., and Walter, P. (1992) Mol. Biol. Cell 3, 895–911
11. Pohlschroder, M., Prinz, W. A., Hartmann, E., and Beckwith, J. (1997) Cell 91, 563–566
12. Oesterhelt, D., and Stoeckensies, W. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 2853–2857
13. Oesterhelt, D. (1998) Curr. Opin. Struct. Biol. 8, 499–500
14. Mathies, R. A., Lin, S. W., Ames, J. B., and Pollard, W. T. (1991) Annu. Rev. Biophys. Biophys. Chem. 20, 491–518
15. Lucke, H., Richter, H. T., and Lanyi, J. K. (1998) Science 280, 1934–1937
16. Pebay-Peyroula, E., Rummel, G., Rosenbusch, J. P., and Landau, E. M. (1997) Science 277, 1676–1681
17. Krebs, M. P., Spudich, E. N., Khorana, H. G., and Spudich, J. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3486–3490
18. Lanyi, J. K., and Oesterhelt, D. (1982) J. Biol. Chem. 257, 2674–2677
19. Schober, B., and Lanyi, J. K. (1982) J. Biol. Chem. 257, 10306–10313
20. Gropp, B., Gropp, F., and Betlach, M. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1204–1208
21. Dale, H., and Krebs, M. P. (1999) J. Biol. Chem. 274, 22693–22698
22. Mevarech, M., and Wertzberger, R. (1985) J. Bacteriol. 162, 461–462
23. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Cline, S. W., Lam, W. L., Charlebois, R. L., Schalkwyk, L. C., and Doolittle, W. F. (1989) Can. J. Microbiol. 35, 148–152
25. Lauring, B., Kreibich, G., and Weidmann, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9435–9439
26. Ferrando, E., Schweiger, U., and Oesterhelt, D. (1993) Gene (Amst.) 123, 41–47
27. Patenge, N., and Soppa, J. (1999) FEMS Microbiol. Lett. 171, 27–35
28. Zentel, T., Roesnshine, J., Betlach, G., Jannasch, H. W., and Levisk, B., and Mevarech, M. (1989) J. Biol. Chem. 264, 18878–18883
29. Pfeifer, F., Grigff, J., and Oesterhelt, D. (1993) Mol. Gen. Genet. 239, 66–71
30. Holmes, M. L., Nuttall, S. D., and Dyall-Smith, M. L. (1991) J. Bacteriol. 173, 3807–3813
31. Mora, E., Lapido, G., Gorkov, D., Lamed, R., Vlachko, M., Bayer, E. A., and Shoham, Y. (1995) Appl. Environ. Microbiol. 61, 1980–1986
32. Nomura, S., Kajimura, N., Matoba, M., Miyata, K., Ortenberg, R., Mevarech, M., Kamikubo, H., Kataoka, M., and Harada, Y. (1999) Langmuir 15, 214–229.
33. Nomura, S., and Harada, Y. (1998) FEMS Microbiol. Lett. 167, 287–293
34. Turner, G. J., Miercke, L. J., Mitra, A. K., Stroud, R. M., Betlach, M. C., and Winter-Vann, A. (1999) Protein Expression Purif. 17, 324–338
35. Manoil, C., and Traxler, B. (1995) Annu. Rev. Genet. 29, 131–150
36. Stewart, C., Bailey, J., and Manoil, C. (1995) J. Biol. Chem. 270, 28078–28084
37. Mingarro, I., Elofsson, A., and von Heijne, G. (1997) J. Mol. Biol. 262, 633–641
38. Fleming, K. G., Ackerman, A. L., and Engelman, D. M. (1997) J. Mol. Biol. 272, 266–275
39. Huang, K. S., Bayley, H., Liao, M. J., London, E., and Khorana, H. G. (1981) J. Biol. Chem. 256, 3802–3809
40. Marti, T. (1998) J. Biol. Chem. 273, 9312–9322
41. Ridge, K. D., Lee, S. S., and Yao, L. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3204–3208
42. Ridge, K. D., Lee, S. S., and Abdalaev, N. G. (1996) J. Biol. Chem. 271, 7860–7867
43. Bibi, E., and Kaback, H. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3204–3208
44. Groves, J. D., and Tanner, M. J. (1995) J. Biol. Chem. 270, 9097–9105
45. Wang, L., Groves, J. D., Mawby, W. J., and Tanner, M. J. (1996) J. Biol. Chem. 272, 10631–10638
46. Koebnik, R. (1996) EMBO J. 15, 3529–3537
47. Engelman, D. M., and Zaccai, G. (1989) Proc. Natl. Acad. Sci. U. S. A. 77, 5894–5898
48. Baker, E. K., Colley, N. J., and Zuker, C. S. (1994) EMBO J. 13, 4886–4895
49. Perazzona, B., Spudich, E. N., and Spudich, J. L. (1996) J. Bacteriol. 178, 6475–6478
50. Bohkareva, E., Seluanov, A., Bibi, E., and Girshovich, A. (1996) J. Biol. Chem. 271, 22256–22261
51. Chen, G. Q., and Gouaux, J. E. (1996) Protein Sci. 5, 456–467
52. Woelfer, U., Dencher, N. A., Buldt, G., and Wrede, P. (1988) Eur. J. Biochem. 174, 51–57
53. Pugsley, A. P. (1993) Microbiol. Rev. 57, 50–108
54. Pekkes, P., and Driessen, A. J. M. (1999) Microbiol. Mol. Biol. Rev. 63, 161–173
55. Xu, Z.-j., Moffett, D. B., Peters, T. R., Smith, L. D., Perry, B. P., Stolke, S. A., Whitmers, J., and Teintze, M. (1995) J. Biol. Chem. 270, 24858–24863

In Vivo Membrane Insertion of Bacterioopsin