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Characterization of Inverted Membrane Vesicles from the Halophilic Archaeon *Haloferax volcanii*

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Abstract. Membrane-related processes in archaea, the third and most-recently described domain of life, are in general only poorly understood. One obstacle to a functional understanding of archaeal membrane-associated activities corresponds to a lack of archaeal model membrane systems. In the following, characterization of inverted archaeal membrane vesicles, prepared from the halophilic archaeon *Haloferax volcanii*, is presented. The inverted topology of the vesicles was revealed by defining the orientation of membrane-bound enzymes that in intact cells normally face the cytoplasm or of other protein markers, known to face the exterior medium in intact cells. Electron microscopy, protease protection assays and lectin-binding experiments confirmed the sealed nature of the vesicles. Upon alkalinization of the external medium, the vesicles were able to generate ATP, reflecting the functional nature of the membrane preparation. The availability of preparative scale amounts of inverted archaeal membrane vesicles provides a platform for the study of various membrane-related phenomena in archaea.

Key words: Archaea — Inverted membrane vesicles — *Haloferax volcanii* — Membrane proteins — Membrane transport — Halophiles

Introduction

With the proliferating number of completed genome-sequencing projects, it is now possible to compare representatives from each of the three domains of life, i.e., eukarya, bacteria and archaea, at the gene and molecular level. Such comparisons reveal that 25–68% of the open reading frames in archaeal genomes sequenced thus far find no counterparts in either bacteria or eukaryotes (depending on the method of comparison), confirming that archaea indeed represent a unique form of life on the planet [15, 17]. Thus, archaea clearly possess distinct and defining traits, in many cases related to the ability of these microorganisms to survive excesses in temperature, salinity and pH, to grow in sulfur-based environments or to metabolize single carbon sources [24].

Exposed to the outside world, the archaeal plasma membrane has to not only withstand the drastic conditions of the extreme habitats in which archaea thrive, yet must also assume a central role in a variety of prokaryotic membrane-associated activities, including transport, secretion, cell division and bioenenergetic-related processes. Understanding how these membrane-based functions are performed in archaea would help not only to describe strategies employed by these microorganisms in overcoming the physical challenges of their environment, but could also shed new light on the mechanisms involved in the processes themselves.

The ability of archaeal membranes to fulfill a variety of normal cellular functions despite their extreme surroundings can be partially related to the unique phospholipids which make up these structures. Unlike the phospholipids found in eukaryal and bacterial membranes, in which fatty acyl groups are ester-bonded to the *sn*-1,2 carbons of a glycerol backbone, the distinctive phospholipids of archaeal membranes are comprised of repeating isoprenyl groups linked to *sn*-2,3 glycerol carbons or to other alcohols such as nonitol through an ether linkage [23]. Some thermoarchaeal species contain membrane-spanning tetraetheric phospholipids, in which both ends of C_{40} polyprenyl chains are ether-linked to glycerol or nonitol [6]. Studies involving archaeosomes, i.e., liposomes prepared from archaeal phospholipids, have

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shown that membranes containing these chemically unique structures are more rigid, proton and sodium ion impermeant, lipase resistant and better able to withstand extremes of pH, temperature and salinity than membranes prepared from bacterial or eukaryal phospholipids [cf. 34, 41].

Whereas much is known concerning archaeal lipids, considerably less is known about archaeal membrane proteins. Membrane proteins involved in bioenergetic-related processes, protein export and solute transport have been described in archaea, yet numerous questions regarding the function of these components remain unanswered. In part, this is due to difficulties in isolating functional archaeal membrane preparations of known orientation. In the case of *Escherichia coli*, sealed plasma membrane vesicles have been widely employed for the biochemical dissection and in vitro reconstruction of a wide range of membrane-associated phenomena. Depending on the mode of preparation, vesicles of differing topology have been obtained; sonication or use of a French-press pressure cell leads to formation of mostly inverted membrane vesicles (IMVs) [14]. Whereas membrane vesicles possessing an orientation similar to that of intact cells, i.e., right-side out, have been obtained from a variety of archaea, reports of inverted archaeal membrane preparations are more rare [2, 16, 36]. Failure to obtain IMVs from the vast majority of archaeal species in which vesicle preparation was attempted is generally attributed to the presence of a proteinaceous surface (S)-layer, a rigid structure often intimately associated with the plasma membrane [36]. Despite the presence of a protein-based shell comprised of a single glycoprotein species [25, 39], we report successful preparation of large-scale amounts of IMVs from the halophilic archaeon *Haloferax volcanii*. In the following, these vesicles are characterized in terms of membrane orientation, sealing and functional behavior.

Materials and Methods

Materials

2,2′-Azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS), ADP, ATP, bovine serum albumin (BSA), carboxyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP), horseradish peroxidase (HRP)-conjugated concanavalin A (ConA), luciferase, malachite green, menadione (2-methyl-1,4-naphthoquinone), 2-(N-morpholino)ethanesulfonic acid (MES), NADH, perchloric acid, periodic acid, 1,4-piperazinediethanesulfonic acid (PIPES) and Schiff reagent were obtained from Sigma (St. Louis, MO). Firefly-D-luciferin was purchased from Biosynth AG (Staad, Switzerland). Proteinase K came from Boehringer-Mannheim (Mannheim, Germany). Tryptone came from US biochemicals (Cleveland, OH) while yeast extract came from Becton Dickinson (Cockeysville, MD). *Haloferax volcanii* DS2 was obtained from the American Tissue Culture Collection and grown aerobically at 40°C as previously described [31].

Preparation of IMVs

To prepare IMVs, cells (5 liter) were grown to mid-exponential phase and harvested by centrifugation (30 min, 8, 000 × g). The cells were resuspended in 50 ml of buffer A (1.75 M NaCl, 50 mM Tris-HCl, pH 7.2) to an OD_{520} of approximately 40 and ruptured with a French Press pressure cell (3 passes at 8, 000 psi each). Cell debris was removed by centrifugation (8, 000 × g, 20 min). The supernatant was then centrifuged in a Beckman L7-80 ultracentrifuge (60-Ti rotor, 50, 000 rpm, 60 min, 4°C). The resulting pellet was resuspended in 5 ml of buffer A and 1 ml aliquots were applied to each of four step gradients prepared with 0.9, 1.1., 1.2, 1.3, 1.4 and 1.5 M sucrose in buffer B (2 M NaCl, 50 mM Tris-HCl, pH 7.2). Upon centrifugation (SW-28 rotor, 18, 000 rpm, 40 hr, 4°C), four distinct, red-colored bands appeared. Each gradient band was collected, diluted with 20 ml buffer B, centrifuged (60-Ti rotor, 45, 000 rpm, 1 hr, 4°C) and the resulting pellets were resuspended in 0.5 ml buffer B, aliquoted and frozen in liquid N_2. The aliquots were stored at −70°C until assayed. The membrane fractions retained their enzymatic activities for up to 3 months under these storage conditions.

Menadione-dependent NADH Dehydrogenase Activity

Menadione-dependent NADH dehydrogenase activity was measured essentially as described previously [12]. Various *H. volcanii* membrane preparations (0.2 mg/ml) were suspended in 2 M NaCl, 0.5 mM MES, 0.5 mM PIPES, pH 6.5. Menadione (0.5 mM) was added to the sample and the reaction was initiated upon addition of NADH (0.5 mM). OD_{340} was then determined at 10-sec intervals. In some cases, the membranes were incubated with 0.01% (w/v) Triton X-100 (60 min, RT) prior to addition of menadione and NADH.

ATPase Activity

Various *H. volcanii* preparations (0.16 mg/ml) were added to a mixture containing 5 mM ATP, 2 M NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 9.5 [16] and transferred to 40°C. At 15-min intervals, 30 μl aliquots were removed and combined with 800 μl Malachite green solution [26]. After 5 min, 100 μl of 34% (w/v) citric acid was added. 30 min later, the absorbance of the samples at OD_{660} was determined. In control experiments, either ATP or membrane-containing fractions were omitted.

Electron Microscopy

H. volcanii preparations were processed and examined by electron microscopy essentially as described previously [37]. Aliquots of the various preparations were fixed with an equal volume of 5% glutaraldehyde in buffer C (2 M NaCl, 0.01 M $CaCl_2$, 0.1 M cacodylate, pH 7.1). After 1 hr on ice, the samples were washed in buffer C and returned to ice for an additional hour. These steps were repeated two more times. The samples were then incubated with 1% osmium tetraoxide in buffer C (overnight, 4°C). Following washes in buffer C (3 × 0.5 ml), the fixed crude IMV and sucrose-gradient band preparations (but not the intact cells) were embedded in 2% (w/v) agar in buffer C. The samples were then cut into small pieces, stained with 1% uranyl acetate, serially dehydrated with 30–100% ethanol, transferred to propylene oxide and embedded in araldite. Thin sections were cut, collected on copper grids, stained with uranyl acetate (30 min) and lead citrate (2 min) and examined in a JEOL 100-B electron microscope.

Lectin-binding Studies

The lectin-binding behaviour of intact cells or sucrose-gradient band 2 membranes was tested by adding HRP-conjugated ConA (10 μg/ml) to membranes (0.2 mg/ml) in 2 M NaCl, 1 mg/ml BSA, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.2 for 1 hr at room temperature. Unbound lectin was then removed by applying the samples to a sucrose gradient prepared with steps of 0.7 and 0.9 M sucrose in buffer B followed by centrifugation in a Beckman TLX Optima tabletop ultracentrifuge (TL 100.2 rotor, 73, 000 rpm, 30 min, 4°C). Pellets were resuspended in 1 ml of ABTS solution (1.8 mM ABTS, 44 mM citric acid, 28 mM Na_2HPO_4) containing 6 μl DNase (1 mg/ml). H_2O_2 was added to a final concentration of 0.003% (v/v) and the samples were nutated at room temperature. After 1 hr, the absorbance of the samples at OD_{410} was determined. In control experiments, either 0.3 M sucrose was added during incubation with the lectin or HRP-conjugated ConA was omitted from the reaction.

ATP Synthesis

The ATP synthesizing ability of the membranes was measured essentially as described previously [38]. Membranes (100 μg) were transferred into 700 μl of a mixture containing (in mM) 50 NADH, 20 $MgCl_2$, 5 Na_2HPO_4, 5 ADP, 50 Tris-HCl, pH 9, and 1.75 M NaCl. The reaction mixture was transferred to 40°C and at various intervals, 100 μl aliquots were removed and transferred into an equal volume of perchloric acid (0.5 M final concentration). The samples were immediately neutralized upon addition of 200 μl of 1 M KOH, stored on ice for 20 min and subjected to centrifugation in a microfuge (6, 000 rpm, 15 min, 4°C). The ATP concentration of the supernatant was determined by a luciferin/luciferase assay. Briefly, 50 μl of the supernatant were added to 500 μl of 2 mM EDTA, 5 mM $MgSO_4$, 10 mM K_2SO_4, 100 mM Tris-acetate, pH 7.75. Luciferin and luciferase were then added and the luminescence of the reaction was measured in an LKB 1250 luminometer (Uppsala, Sweden).

Other Methods

Protein concentration was determined by Bradford reagent (BioRad, Hercules, CA), using BSA as a standard. SDS-PAGE was performed using 15 or 7.5% gels. Immunoblotting was performed using antibodies raised against *E. coli* trigger factor [19] or *H. volcanii* dihydrofolate reductase (DHFR). Antibody binding was detected using goat anti-rabbit HRP-conjugated antibodies (BioRad) and an ECL kit (Amersham, Buckingham, UK). Periodate-Schiff reagent (PAS) staining was performed as described previously [9]. Spectroscopy was performed using a Novaspec II spectrophotometer (Pharmacia, Uppsula, Sweden). Densitometry was performed using a UMAX Mirage IIse scanner (Willich, Germany) and IPLab Gel software (Signal Analytics, Vienna, VI).

Results

Separation of *H. volcanii* Membrane Preparations on Sucrose Step Gradients

As a first step in the preparation of *H. volcanii* IMVs, cells were grown to mid-exponential phase and disrupted using a French press pressure cell. In earlier experiments, cells were ruptured by sonification, but this approach yielded far less consistent results than achieved using the French press (*not shown*). The membrane preparation obtained following cell disruption with the French press, referred to as the crude IMV preparation, theoretically contains a mixture of intact cells, right-side out vesicles, IMVs and membrane fragments. To separate the various membrane fractions contained within the crude IMV preparation, centrifugation on sucrose density gradients comprised of 0.9, 1.1, 1.2, 1.3, 1.4 and 1.5 M sucrose steps prepared in high salt-containing buffer was performed. Preliminary experiments addressed the behavior of defined membrane preparations on such gradients. Centrifugation of intact cells led to the appearance of a pink film at the surface of the gradients and a red pellet at the bottom of the gradients (*not shown*). Cells osmotically lysed by transfer into water yielded a similar pattern, in addition to the appearance of a red-pink band in the lower third of the gradients. A more complex pattern was obtained when the French press-disrupted crude IMV preparation was applied to the sucrose density gradients (Fig. 1). Following centrifugation, four distinct bands were clearly discerned. Band 1 was found at the surface of the gradients, band 2 collected at the 1.1–1.2 M sucrose steps interface, band 3 appeared at the 1.3–1.4 M sucrose steps interface, while band 4 consisted of a small pellet at the base of the gradient tubes. Following collection and recentrifugation, pellets obtained from each of the four sucrose gradient bands were resuspended in sucrose-free, high salt-containing buffer and tested for the presence of inverted, sealed and functional membrane vesicles.

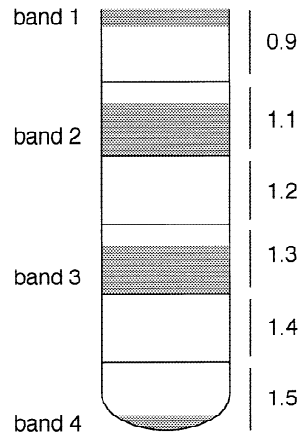

Fig. 1. Schematic diagram of the crude IMV preparation following centrifugation on sucrose density gradients. As described in Materials and Methods, an aliquot of the crude IMV preparation (16.0 mg/ml) was applied to a gradient prepared from steps of 0.9 and 1.1–1.5 M sucrose in 2 M NaCl, 50 mM Tris-HCl, pH 7.2 and subjected to ultracentrifugation. The molar concentration of sucrose in each region of the gradient is shown on the right, while the positions of the different bands are shown on the left.

Table 1. Menadione-dependent NADH dehydrogenase activity

Membrane preparation	Specific activity[a]		Percent activity[c]
		+0.01% Triton X-100[b]	
Cells	0 (5)	6.6 ± 0.8 (5)	0
Crude IMVs	2.7 ± 0.5 (5)	3.8 ± 0.8 (5)	71
Sucrose gradient band 1	0 (11)	3.4 ± 0.5 (11)	0
Sucrose gradient band 2	2.2 ± 0.2 (11)	2.4 ± 0.2 (11)	92
Sucrose gradient band 3	0 (11)	1.8 ± 0.2 (11)	0
Sucrose gradient band 4	0 (11)	2.9 ± 0.5 (11)	0

[a] Values are expressed as ΔOD_{340}/min/mg protein ± SEM (number of repeats).
[b] Values measured after 0.01% Triton X-100 pretreatment represent the total enzyme activity in a sample.
[c] Values are expressed as percent of the activity determined after 0.01% Triton X-100 pretreatment, taken as 100% in each case.

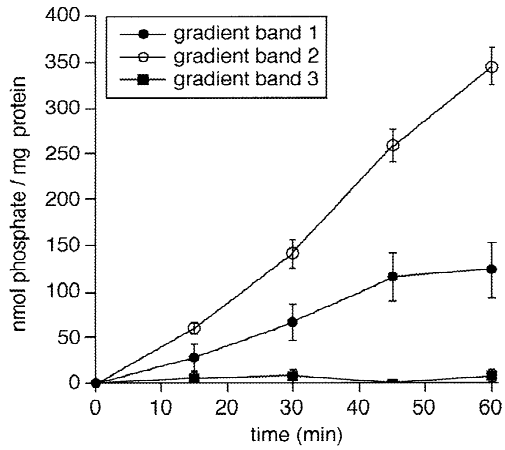

Fig. 2. Sucrose gradient band 2 membranes possess externally-oriented ATPase activity. Aliquots of sucrose gradient bands 1, 2 and 3 were assayed for ATPase activity by spectrophotometrically measuring the amount of liberated orthophosphate. ATPase activities of sucrose gradient band 1 (filled circles), sucrose gradient band 2 (empty circles), and sucrose gradient band 3 (filled squares) are shown. Each point represents the average of 6–9 experiments ± SEM, following corrections for the contribution of spontaneous ATP breakdown.

Sucrose Gradient Band 2 Contains Membranes of Inverted Topology

To address the topological orientation of the membrane populations isolated from the four different regions of the sucrose gradients, each sucrose gradient band was assayed for the activity of membrane-associated enzymes that face the cytoplasm in intact cells, such as menadione-dependent NADH dehydrogenase or membrane-bound ATPase [21, 29]. In intact cells, menadione-dependent NADH dehydrogenase activity could not be detected unless cellular integrity was first compromised by Triton X-100 so as to allow access of the substrate to the enzyme (Table 1). No enzyme activity was measured in samples treated with Triton X-100 in the absence of substrate (*not shown*). Moreover, incubation with a 10-fold higher concentration of detergent did not result in an increase in the level of activity, confirming that the Triton X-100 treatment effectively exposed internally-oriented active sites to substrate, yet did not modulate enzyme activity (*not shown*). Higher Triton X-100 concentrations interfered with the assay measurements. In contrast to results obtained using intact cells, assaying crude IMVs revealed that 71% of the total menadione-dependent NADH dehydrogenase activity was exposed to the medium, with the activity measured following Triton X-100 pretreatment considered as 100%. When comparable amounts of membranes obtained from the different sucrose gradient bands (as determined by protein concentration) were tested for menadione-dependent NADH dehydrogenase activity, no activity could be detected in sucrose gradient bands 1, 3 or 4 unless the membranes had been pretreated with detergent. In the case of sucrose gradient band 2, 92% of the total menadione-dependent NADH dehydrogenase activity could be detected prior to addition of detergent. Thus, as judged by menadione-dependent NADH dehydrogenase activity, membranes in sucrose gradient band 2 display inverted topology. This assay further suggests that separation of sucrose gradient band 2 membranes from the crude IMVs results in a 1.3-fold enrichment of the inverted membrane pool.

Determination of membrane-associated ATPase activity was also used as a marker of membrane topology. In a variety of haloarchaea, including *H. volcanii*, internally-oriented membrane-bound ATPases have been described and extensively characterized in terms of kinetics, cofactor requirements and inhibitor sensitivities [4, 10, 22, 33]. The enzymatic hydrolysis of externally added ATP can be measured colorimetrically in an assay which reflects the concentration of liberated orthophosphate [26]. In the case of *H. volcanii* membrane preparations, negligible ATP hydrolysis was detected in reactions containing intact cells, whereas crude IMVs were able to readily hydrolyze ATP, confirming that significant inversion of membrane topology had been achieved in the crude IMV preparation (*not shown*). Examination of the ATPase activity of the different sucrose gradient bands revealed the presence of substantial ATP-hydrolyzing activity in sucrose gradient band 2 (Fig. 2). Sucrose gradient band 1 membranes possessed less than 30% of that activity, while only negligible ATPase activity was detected in sucrose gradient band 3. In the absence of either ATP or membranes, only minor changes in orthophosphate levels could be detected (*not shown*). Thus, in agreement with results of the menadione-dependent NADH dehydrogenase activity assay, the

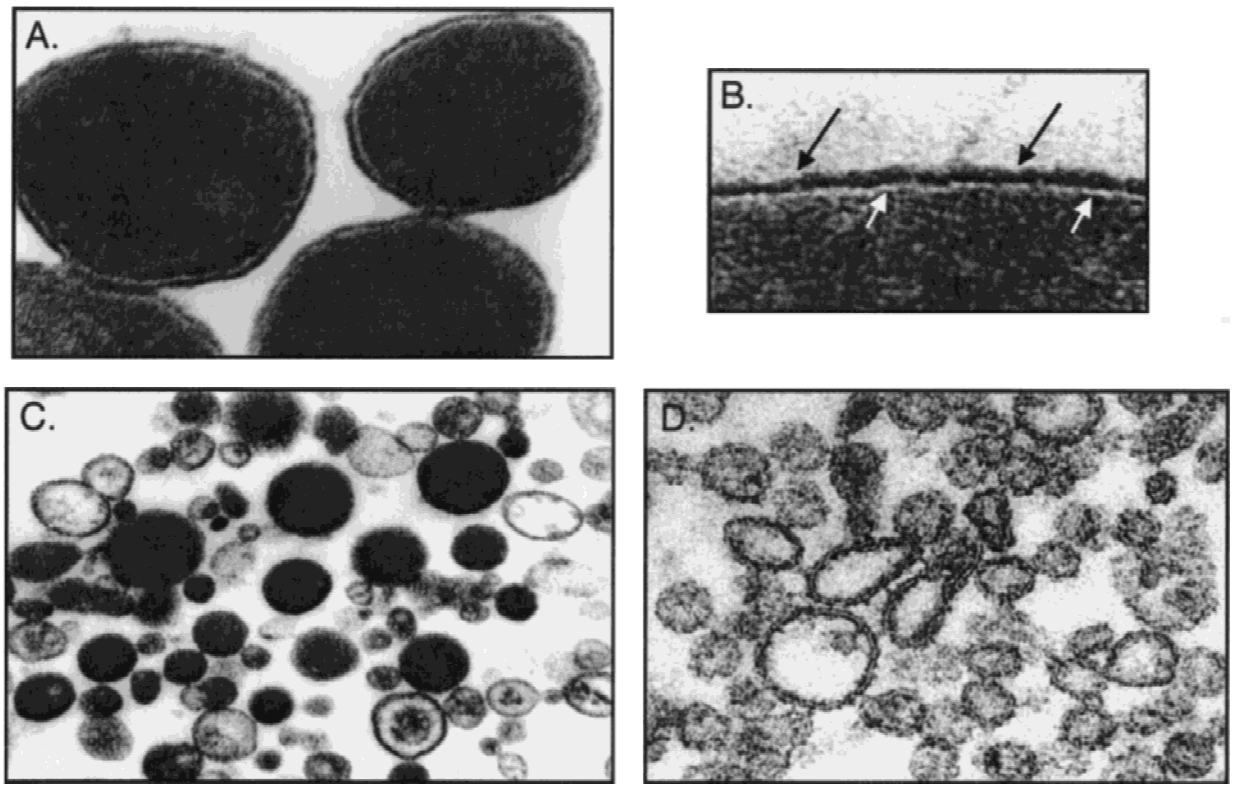

Fig. 3. Electron microscopic examination of different *H. volcanii* membrane preparations. (*A*) Intact cells (magnification ×140, 000). (*B*) Enlarged cell border region. Black arrows show the S-layer, white arrows show the plasma membrane (magnification ×228, 000). (*C*) crude IMV preparation (magnification ×31, 000). (*D*) sucrose gradient band 2 (magnification ×107, 000).

ATPase activity of sucrose gradient band 2 also supports the assigned inverted topology of this membrane fraction.

Band 2 Membranes Exist as Sealed Vesicles

Since the active sites of normally cytoplasmic-facing enzymes would be exposed to the external milieu in membrane preparations containing IMVs and/or membrane fragments, a series of experiments were next undertaken to confirm the sealed nature of the membrane preparation isolated in sucrose gradient band 2.

The sealed nature of sucrose gradient band 2 membranes was first investigated by electron microscopy. Preliminary experiments involving intact cells were performed to confirm that the protocol employed for sample preparation did not compromise *H. volcanii* membrane integrity or ultrastructure. As shown in Fig. 3*A* and highlighted in Fig. 3*B*, the cytoplasm of intact cells displays a defined border (*white arrows*, Fig. 3*B*) surrounded by a narrow band of 'periplasmic' space enclosed by an intact and continuous S-layer cell border (*black arrows*, Fig. 3*B*), as previously reported [28]. Electron microscopic examination of structures present in the crude IMV preparation revealed the presence of sealed membrane vesicles of differing sizes, as well as intact cells and various cellular fragments (Fig. 3*C*). Significantly more sealed and empty membrane vesicles were observed in samples prepared from sucrose gradient band 2 (Fig. 3*D*). Examination of the border of these vesicles revealed the presence of a double membrane structure, most likely consisting of the isolated plasma membrane and the attached proteinaceous S-layer. The presence of the S-layer glycoprotein in sucrose gradient band 2 membranes was confirmed in SDS-PAGE experiments (*see* below). Sealed vesicle formation was quantitated by examining micrographs of cells, crude IMVs and sucrose gradient band 2 membranes and expressing the proportion of cytoplasm-lacking, sealed membrane vesicles counted as a function of the total number of intact membrane-enclosed structures (Fig. 4). In the case of intact cells, no cytoplasm-lacking vesicles were detected. Examination of the crude IMV preparation revealed that approximately 30% of the structures appeared as sealed and empty vesicles. When sucrose gradient band 2 was similarly examined, over 70% of the structures observed were in the form of empty, sealed vesicles, ranging from 0.15–0.25 μm in diameter. The non-sealed structures detected in the sucrose gradient

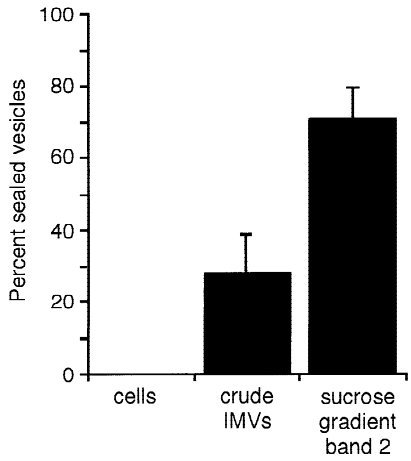

Fig. 4. Quantitation of sealed-vesicle formation. The percentage of sealed vesicles relative to the total number of membrane-enclosed structures is portrayed. Values shown represent the average percentage of sealed vesicles counted in the micrographs of a particular membrane preparation ± standard deviation, obtained from 2 experiments. For intact cells, a total of 190 structures was examined. In the case of crude IMVs, a total of 350/1151 (30.4%) structures was in the form of empty, sealed vesicles, while in sucrose gradient band 2, a total of 483/675 (71.6%) structures appeared as empty and sealed membrane vesicles.

band 2 sample could correspond to vesicles that became ruptured during the fixation phase of EM sample preparation.

The sealed and inverted nature of the vesicles was also shown by determining the accessibility of cellular membrane markers to added protease. The *H. volcanii* S-layer glycoprotein is found on the external face of the plasma membrane, apparently linked to the membrane via a stretch of hydrophobic amino-acid residues located near the C-terminus of the protein [39]. In vesicles of inverted topology, i.e., IMVs, the S-layer glycoprotein would face the vesicle interior and hence be inaccessible to externally added protease. In contrast, menadione-dependent NADH dehydrogenase would be exposed to the surrounding medium in IMVs, as discussed above. To confirm the sealed and inverted nature of a given membrane preparation, proteinase K was added and access of the protease to the S-layer glycoprotein and the menadione-dependent NADH dehydrogenase was determined. When the membrane preparation from sucrose gradient band 2 was examined following proteolysis of 4 hr, only 21% of the starting externally-oriented menadione-dependent NADH dehydrogenase activity remained (Fig. 5*A*). A proportional drop in the starting total enzyme activity, revealed following detergent-induced membrane dissolution, was also detected. In contrast to the readily digested menadione-dependent NADH dehydrogenase activity in sucrose gradient band 2, 94% of the S-layer glycoprotein in the same membrane preparation was protected from proteolytic digestion, as revealed by SDS-PAGE and densitometry (Fig. 5*C*). When similar experiments were performed on sucrose gradient band 3 membranes, the opposite situation was observed, presumably due to the cell-like orientation of these membranes (*see* above). As such, no menadione-dependent NADH dehydrogenase activity was detected either before or after protease treatment in the absence of Triton X-100, as expected. Upon disruption of membrane integrity by detergent treatment, all of the starting enzymatic activity could be detected even after a 4 hr incubation with proteinase K, implying protection of the enzyme from the proteolytic treatment (Fig. 5*B*). Significant digestion of the S-layer glycoprotein was, however, detected in the intact sucrose gradient band 3 membrane preparation. Within 4 hr of proteolysis, less than 20% of the starting S-layer glycoprotein content remained, as determined densitometrically (Fig. 5*C*). In control experiments, membranes from both sucrose gradient bands were incubated at 40°C for up to 4 hr in the absence of protease, during which time no losses in either menadione-dependent NADH dehydrogenase activity nor S-layer glycoprotein content were detected, revealing the absence of any endogenous proteolytic activity (*not shown*).

The sealed nature of the membrane-vesicle preparation isolated in sucrose gradient band 2 was further shown by addressing the binding of HRP-conjugated ConA, via interaction of the lectin with the S-layer glycoprotein or other externally-oriented glycan-containing structures, such as glycolipids [42]. As the major glycosylated protein species in *H. volcanii*, the S-layer glycoprotein has been previously shown to specifically interact with ConA [11]. Incubation of HRP-conjugated ConA with intact cells and subsequent detection of lectin binding via colorimetric detection of the linked HRP activity revealed the ability of ConA to bind to externally-oriented glycan structures, as expected (Table 2). The interaction of ConA with the cells was significantly reduced upon addition of excess glucose, due to competition for lectin binding between the added sugar and cell surface glycan moieties. Since enzymatic activity could be readily detected in these control experiments, it can be concluded that transient exposure of the HRP moiety to high salt conditions during incubation with the cells did not lead to an irreversible loss of enzymatic activity. When the ability of the lectin to bind to sucrose gradient band 2 membrane vesicles was tested, less than 20% of the binding measured with intact cells was detected, suggesting that externally-oriented lectin binding targets were largely inaccessible in the membrane vesicles. To confirm that the preparation of sucrose gradient band 2 membrane vesicles did not result in a loss of glycosylated membrane-associated elements, the glycoprotein contents of the intact cells and sucrose gradient band 2 membrane vesicles were examined by SDS-PAGE and PAS staining and quantitated densitometrically. In both

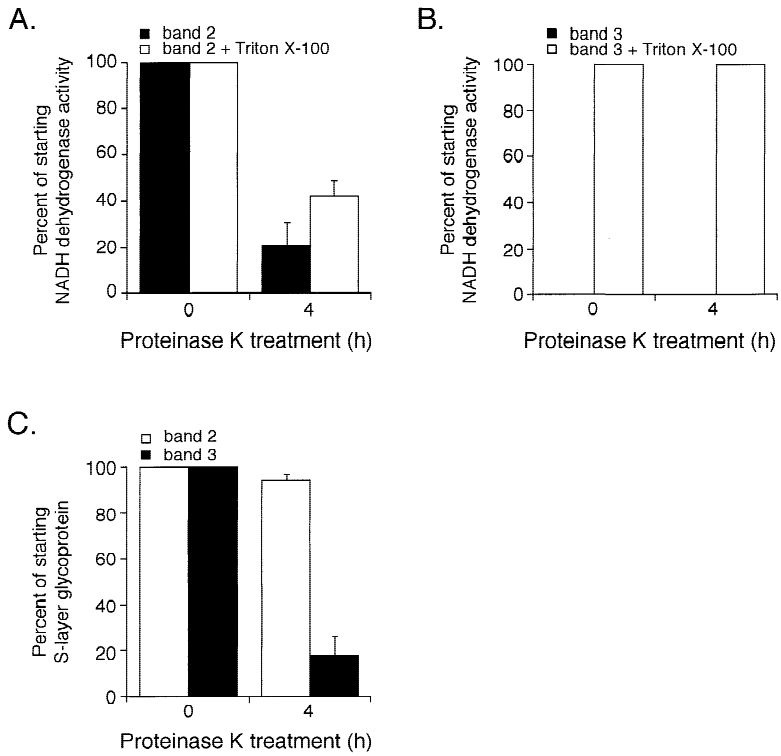

Fig. 5. Protease accessibility reveals the sealed nature of sucrose gradient band 2 membranes. Samples of sucrose gradient band 2 and band 3 (0.7 mg each) were incubated with 1 mg/ml proteinase K at 40°C. Aliquots were removed immediately prior to protease addition (t = 0) and 4 hr after the onset of proteolysis (t = 4) and examined as follows: 100 μl portions of each membrane fraction were assayed for menadione-dependent NADH dehydrogenase activity in either the presence or absence of 0.01% Triton X-100, as described in the Materials and Methods section, except that the period of incubation with the detergent was shortened to 15 min. In addition, aliquots of the sucrose gradient band 2 and band 3 membranes were removed at times t = 0 and t = 4, precipitated with 15% TCA and processed for SDS-PAGE. Following Coomassie staining, the level of the S-layer glycoprotein in each sample was determined densitometrically. The levels of menadione-dependent NADH dehydrogenase activity or S-layer glycoprotein present at a given time point were expressed as a percentage of those levels detected prior to protease treatment, taken as 100%. (*A*) Menadione-dependent NADH dehydrogenase activity of sucrose gradient band 2 membranes, measured prior to (black bars) and following (white bars) incubation with 0.01% Triton X-100. (*B*) Menadione-dependent NADH dehydrogenase activity of sucrose gradient band 3 membranes, presented as in *A*. (*C*) S-layer glycoprotein levels in sucrose gradient band 2 (white bars) and band 3 (black bars) membranes. In each panel, each bar represents the average of 3–5 experiments ± SEM.

Table 2. Binding of HRP-conjugated ConA

Treatment	Intact cells[a]	Sucrose gradient band 2
Control	0.192	0.037
+0.3 M glucose	0.063	0.031
−HRP-conjugated ConA	0.026	0.008

[a] Values correspond to the average of OD_{410} readings of duplicate samples measured in one of five repeats of the experiment.

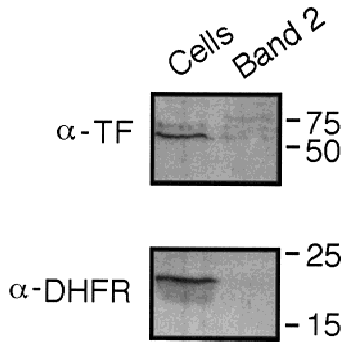

Fig. 6. Sucrose gradient band 2 membrane vesicles are devoid of cytoplasm. Aliquots of intact *H. volcanii* cells and sucrose gradient band 2 membranes containing equal amounts of protein (10 μg) were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies to *E. coli* trigger factor (*upper panel*) or *H. volcanii* DHFR (*lower panel*). Molecular weight markers are shown to the right of each panel.

cases, similar levels of S-layer glycoprotein were detected (*not shown*).

Finally, to confirm that the sucrose gradient band 2 membrane vesicles were devoid of cytoplasm, aliquots of intact cells and sucrose gradient band 2 membranes containing equal amounts of protein were probed in immunoblot experiments using antibodies raised against two cytoplasmic markers, DHFR and trigger factor. *H. volcanii* DHFR has been previously cloned, sequenced and purified [43]. In contrast, trigger factor, a protein shown to be associated with nascent polypeptides in *E. coli* [20], has not been reported in archaea. As expected, anti-DHFR recognized an approximately 23 kDa protein in intact *H. volcanii* cells. No such band was labelled in the sucrose gradient band 2 membranes (Fig. 6, *lower panel*). When immunoblotting was performed using antibodies raised to bacterial trigger factor, labelling of an approximately 60 kDa protein in intact cells was observed (Fig. 6, *upper panel*). As with the anti-DHFR antibodies, no labelling of sucrose gradient band 2 membranes with the anti-trigger factor serum was observed.

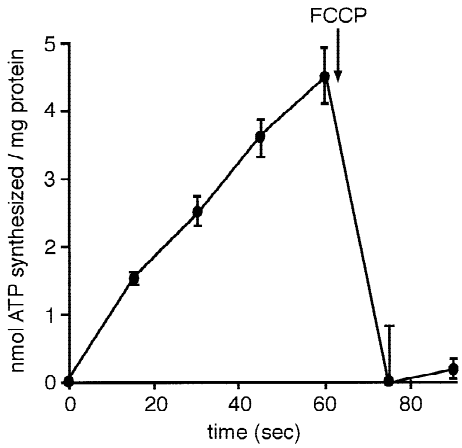

Fig. 7. Sucrose gradient band 2 membranes are capable of synthesizing ATP. The ability of sucrose gradient band 2 membranes (100 μg) to synthesize ATP was revealed by the luciferin/luciferase assay, as described in the Materials and Methods section. After 60 sec of ATP synthesis, 200 μM FCCP was added to the reaction (*arrow*). The data shown represent the average of duplicate samples from one of three experiments ± standard deviation.

Thus, the sucrose gradient band 2 membrane vesicles do not include cytoplasmic markers.

The Inverted Membrane Vesicles in Sucrose Gradient Band 2 are Functional

To determine whether the inverted and sealed membrane vesicles contained in sucrose gradient band 2 were functional in terms of membrane-related activity, the ability of the IMVs to synthesize ATP was tested. IMVs equilibrated in pH 7.2 buffer were placed into a pH 9 environment containing ADP and orthophosphate as well as NADH, an electron donor. At 15-sec intervals, aliquots were removed and tested for the presence of ATP. As revealed in Fig. 7, a linear increase in ATP levels was detected over the course of the reaction. Addition of 200 μM FCCP, an uncoupler of oxidative phosphorylation previously shown to interfere with ATP synthesis in *H. volcanii* [38], led to a rapid drop in ATP levels (Fig. 7, *arrow*). When either ADP or orthophosphate were omitted from the reaction, neglible levels of ATP were detected (*not shown*). These results reveal that the IMVs found in sucrose gradient band 2 are able to establish a proton gradient across the membrane and then exploit this proton gradient to drive a biologically relevant process, in this case, ATP synthesis.

Discussion

The availability of membrane vesicle preparations of different topological orientation has greatly advanced mechanistic understanding of a variety of bacterial and eukaryotic membrane-related cellular processes. For better characterization of such processes in archaea, the most recently described yet least understood domain of life, the availability of pure, sealed membrane vesicle preparations of known orientation is required. With its relatively simple growth requirements, the haloarchaeon *H. volcanii* has proven its applicability in various molecular biology and protein chemistry studies [5]. To extend the usefulness of this species to the study of archaeal membrane biology, the present report describes the large-scale preparation and characterization of IMVs from *H. volcanii.*

Relying on an experimental approach originally designed for the preparation of IMVs from *E. coli* [3, 8], a haloarchaeal membrane preparation was isolated followed cell disruption with a French press pressure cell and centrifugation on sucrose density step gradients. The outward exposure of enzyme activities which face the cytoplasm in the intact cell, protease protection assays, electron microscopy, lectin accessibility as well as ATP-synthesizing ability support the conclusion that sucrose gradient band 2 contains inverted, sealed and functional membrane vesicles. Based on enzymatic activities and electron microscopy analysis, it appears that 70–90% of the membranous structures in sucrose gradient band 2 are IMVs.

While sucrose gradient bands 1, 3 and 4 were not characterized to the same degree as sucrose gradient band 2, the topology of membranes in some of these fractions can be inferred. As judged by enzyme topologies, membranes in sucrose gradient bands 1, 3 and 4 maintain right-side topology. Based upon its behavior in sucrose density gradient centrifugation, sucrose gradient band 4 contains intact cells. Electron microscopy analysis of sucrose gradient band 1 revealed the presence of empty membrane vesicles (*not shown*). It seems, therefore, that sucrose gradient band 1 contains right side-out vesicles. However, due to the small amounts of sucrose gradient band 1 present in the sucrose density gradients, only limited characterization of the membranes of this fraction was possible.

Examination of the literature reveals earlier attempts at preparing membrane vesicles from *H. volcanii.* In the original report of the strain, it was shown that disruption of the plasma membrane by osmotic lysis upon transfer of cells into water yielded small, sealed membrane vesicles [32]. Unfortunately, the topological orientation and preservation of membrane protein function in these vesicles was not determined. More recently, the ability of an *H. volcanii* membrane preparation to synthesize ATP from ADP and orthophosphate upon alkalinization of the medium was reported [38]. While these studies reported a high degree of exposure of the inner face of the membrane to the external medium, no effort to dif-

ferentiate between membrane fragments and sealed vesicles was made, nor was separation of different vesicle populations attempted. The current study, therefore, represents the first complete protocol for the preparation of large quantities of sealed IMVs and subsequent analysis of vesicles prepared according to this protocol.

The presence of a crystalline S-layer apparently does not interfere with the ability of *H. volcanii* membrane fragments to reseal in an inverted manner. Previous attempts at preparation of archaeal IMVs have met with mixed success, apparently due to the presence of a protein-based surface layer. Indeed, preparation of IMVs from *Methanosarcina mazei*, one of the two archaeal strains from which IMVs are currently available, involves prior proteolytic digestion of surface structures [2, 30]. The S-layer glycoprotein has also been cited as the agent responsible for the inability to obtain IMVs from other archaeal strains, including *Sulfolobus acidocaldarius* [28, 36]. In contrast, the proteinaceous S-layer did not prevent the formation of IMVs from *Halobacterium halobium* (*salinarum*) [16, 27]. Three-dimensional reconstructions of the S-layers of *H. volcanii, H. salinarum* and *Sulfolobus* species reveal similar architecture, i.e., a hexagonal lattice comprised of dome-like units separated from the underlying plasma membrane by spacers to create a pseudo-periplasmic space [1, 7, 25, 35, 40]. The proposed pseudo-periplasmic space is, however, thought to be much wider in the case of *Sulfolobus* strains as compared to the haloarchaeal strains. Such differences could affect the relative ease of obtaining IMVs from the haloarchaeal species. Furthermore, while the *H. salinarum* and *H. volcanii* S-layer glycoproteins are believed to be directly anchored into the plasma membrane [27, 39], additional proteins are proposed to be responsible for the anchoring of the S-layer glycoprotein in *Sulfolobus* [1, 18]. Although it is not known how the presence of rigid S-layers affects archaeal membrane lipid behavior [13], the suggested differences in haloarchaeal and *Sulfolobus* S-layer glycoprotein attachment could affect the ability of the membranes to reseal in an inverted manner.

The availability of inverted *H. volcanii* membrane vesicles will prove useful for structural, functional and mechanistic analysis of archaeal membrane-bound enzyme activities. Such studies will further our understanding of membrane-linked processes in halophiles in particular, and in archaea in general. The observation that the S-layer glycoprotein is maintained and protected from proteolysis in the IMVs suggests the usefulness of such vesicles in studies in S-layer glycoprotein translocation and glycosylation.

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