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Lipid membranes from halophilic and alkali-halophilic Archaea have a low H^+ and Na^+ permeability at high salt concentration

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Abstract The influence of pH and the salt concentration on the proton and sodium ion permeability of liposomes formed from lipids of the halophile *Halobacterium salinarum* and the haloalkaliphile *Halorubrum vacuolatum* were studied. In contrast with liposomes formed from *Escherichia coli* lipids, liposomes formed from halophilic lipids remained stable up to 4 M of NaCl and KCl. The proton permeability of the liposomes from lipids of halophiles was independent of the salt concentration and was essentially constant between pH 7 and pH 9. The sodium ion permeability increased with the salt concentration but was 10- to 100 fold lower than the proton permeability. It is concluded that the membranes of halophiles are stable over a wide range of salt concentrations and at elevated pH values and are well adapted to the halophilic conditions.

Key words Membrane stability · Alkaliphiles · Halophiles · Permeability

Introduction

Halophiles live at higher salt concentrations than found in seawater (i.e., above 0.5 M). Extreme halophiles, which thrive optimally above 3 M, are mainly but not exclusively aerobic Archaea. Moderate halophiles are mostly bacteria, but some are methanogens (Archaea) or algae (Eukarya). At extracellular osmolarities exceeding that of the cytoplasm, microorganisms accumulate potassium ions or low molecular weight organic solutes (osmolytes). These compounds enable the cell to reduce the water loss, to maintain cell turgor pressure by reducing the osmotic potential between the cell and the environment, and to protect enzymes from the low water activity (Yancey et al. 1982). Although the outside potassium ion concentration may be only a few millimolar, potassium ions may accumulate to levels up to five times the internal sodium ion concentration (Brown 1983).

Alkaliphiles are organisms that thrive at high pH. Most cytoplasmic enzymes of alkaliphiles function optimally near neutral pH (Booth 1985), and these organisms need to maintain intracellular pH at or around neutrality. Alkaliphilic organisms use the electrochemical gradient of H^+ or Na^+ for energy transduction. The electrochemical gradient of H^+ exerts a proton motive force (pmf), which consists of two components, the transmembrane pH gradient (ΔpH), and the transmembrane electrical potential ($\Delta\psi$). In alkaliphiles, the pH outside is alkaline versus inside acid. This reversed ΔpH is compensated by a large $\Delta\psi$, inside negative, resulting in a net pmf that is directed inward (Van de Vossenberg et al. 1998a).

Aerobic alkaliphiles use a Na^+/H^+-antiporter in combination with H^+-coupled respiration to regulate the intracellular pH (Krulwich 1995; Speelmans et al. 1995). Both marine and nonmarine alkaliphiles require Na^+ in the growth medium (Krulwich 1995). For most haloalkaliphiles the outside concentration of sodium is high and the external H^+ concentration low. In these organisms, an electrogenic Na^+–H^+ (Na^+/H^+ ratio <1) antiport reaction drives both the necessary extrusion of sodium ions and the uptake of protons. Such a mechanism would be frustrated by an unidirectional flux of either Na^+ inward or H^+ outward. Therefore, the membrane of haloalkaliphiles must be highly impermeable for both protons and sodium ions.

Previous studies on the proton permeability in archaeal and bacterial membranes have demonstrated that most Bacteria and Archaea adjust the permeability of the cytoplasmic membranes to the growth temperature of the organism (Elferink et al. 1994; Van de Vossenberg et al. 1995). The proton permeability is maintained at a constant level by regulation of the phospholipid composition of the cell membrane as a function of the growth temperature

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(Van de Vossenberg et al., 1999). The question arises how the salt concentration affects the permeability properties of the membrane of halophiles.

In this study we have analyzed the proton and sodium ion permeability of liposomes prepared from lipids extracted from two aerobic extremely halophilic archaea: *Halobacterium* (*Hb.*) *salinarum* and the haloalkaliphile *Halorubrum* (*Hr.*) *vacuolatum* (renamed from *Natronobacterium vacuolatum* (Mwatha and Grant 1993) and compared with liposomes from *Escherichia coli* lipids. *Hr. vacuolatum* grows optimally at pH 9.5. The salt and pH dependency of the ion permeability shows that the membrane of these organisms is very impermeant to H^+ and Na^+, even at high salt concentrations and pH values. These properties of the cytoplasmic membrane permit growth at these extreme environmental conditions.

Materials and methods

Strains and lipids

Cells of *Hr. vacuolatum* were grown in 10 g l^{-1} yeast extract (Difco), 7.5 g l^{-1} casamino acids, 3 g l^{-1} Na_3 citrate·$2H_2O$, 2 g l^{-1} KCl, 1 g l^{-1} $MgSO_4$·$7H_2O$, 0.36 mg l^{-1} $MnCl_2$·$4H_2O$, 50 mg l^{-1} $FeSO_4$·$7H_2O$, 200 g l^{-1} NaCl, and 18.5 g l^{-1} Na_2CO_3 (anhydrous), pH 10–10.5. Cells of *Hb. salinarum* S9 were kindly provided by Dr. H. Bolhuis, Max Planck Institut für Biochemie, Martinsried, Germany. Cells were grown in 250 g l^{-1} NaCl, 20 g l^{-1} $MgSO_4$·$7H_2O$, 3.0 g l^{-1} Na_3 citrate, 2.0 g l^{-1} KCl, and 10 g l^{-1} bactopeptone (Oxoid), pH 7.0–7.2. Lipids were isolated according to Lo and Chang (1990), and stored in chloroform/methanol/water (65/25/4, v/v/v) under a N_2 atmosphere at −20°C.

A total lipid extract of *E. coli* B (ATCC 11303), grown in Kornberg minimal medium (Ashworth and Kornberg 1966), pH 7.4 at 37°C, was obtained from Avanti Polar Lipids (Alabaster, AL, USA). The lipids were acetone and ether washed according to Kagawa and Racker (1971) and stored in chloroform under a N_2 atmosphere at −20°C.

The lipids of *Hb. salinarum* and *Hr. vacuolatum* were analyzed by thin-layer chromatography using Kieselgel 60 (Merck, Darmstadt, Denmark) plates and chloroform/methanol/water (65/25/4, v/v/v) as eluant. Plates were developed with iodine vapor and a molybdate reagent to reveal the presence of (phospho-)lipids (Rouser et al. 1970).

Liposome preparation and integrity determination

Lipids were dried by vacuum rotary evaporation and hydrated in 50 mM 4-morpholine-propane sulfonic acid (MOPS), pH 7.0, 75 mM KCl, and 25 mM choline to a final concentration of 20 mg ml^{-1}, unless indicated otherwise. NaCl or KCl was included in the buffer at the desired concentration. The pH of the medium was varied by the use of 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (pH 7.5), N-tris(hydroxymethyl)methylglycine (TRICINE) (pH 8), or 2-(N-cyclohexylamino) ethane sulfonic acid (CHES) (pH 9). Liposomes were sized by five consecutive freezing and thawing steps, followed by extrusion through 400- and 200-nm polycarbonate filters using the Liposofast (Basic, Avestin, Ottawa, Canada) extrusion apparatus (Elferink et al. 1994). Liposome integrity was tested by the ability to maintain a $\Delta\psi$, formed by a potassium diffusion gradient in the presence of valinomycin. The $\Delta\psi$ (inside negative) was monitored with the fluorescent probe 3,3′-diethylthiadicarbocyanine iodide ($DiSC_3(5)$] (Singh et al. 1985).

Particle sizing of liposomes

The size distribution of the liposomes was determined by photon correlation spectroscopy using a Submicron Particle Sizer model 370 (Nicomp, Santa Barbara, CA, USA) (Payne and New 1990).

Proton and sodium ion permeability

The proton permeability of the liposomes was measured with the acid-pulse method, monitored with fluorescent probes (Molecular Probes, Leiden, The Netherlands), as described by Nichols and Deamer (1980), Elferink et al. (1994), and Van de Vossenberg et al. (1995). The probe 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) (pyranine, $pK_a = 7.3$) was used for proton permeability measurements between pH 7.0 and 8.0, and SNAFL (Molecular Probes, Leiden, The Netherlands) ($pK_a = 7.8$) at pH 9.0. Data were fitted to the first-order kinetic equation, from which the first-order rate constant k_{H+} was used to compare the permeability properties of the membranes.

The sodium ion permeability of the liposomes was estimated from the efflux of ^{22}Na. Liposomes were prepared in buffer A (50 mM MOPS·KOH, pH 7.0, 100 mM KCl) supplemented with NaCl at the concentration indicated. To 100 µl of the liposome suspension, $^{22}NaCl$ (~3×10^5 cpm; specific activity >2200 Ci/mol; Amersham) was added, and allowed to equilibrate by incubation for at least 18 h at room temperature. To initiate ^{22}Na efflux, the liposomal suspension was diluted 100 fold in 10 ml buffer A with the appropriate salt concentration. At various time intervals, 1-ml samples were taken and filtered over a 0.2-µm nitrocellulose filter (Schleicher & Schuell, Dassel, Germany). Filters were rinsed with 1 ml buffer, and the amount of label retained on the filter was determined with a liquid scintillation counter. Data were corrected for the amount of ^{22}Na bound to the filters on the permeabilization of the liposomes with 1 µM of Gramicidin D. Sodium ion efflux was fitted to the first-order kinetic equation as described for proton influx to yield the first-order rate constant for the sodium ion permeability, k_{Na+}.

Results

Liposome integrity and stability

Lipids were extracted from the halophilic archaeon *Hb. salinarum* and the halo-alkaliphilic archaeon *Hr.*

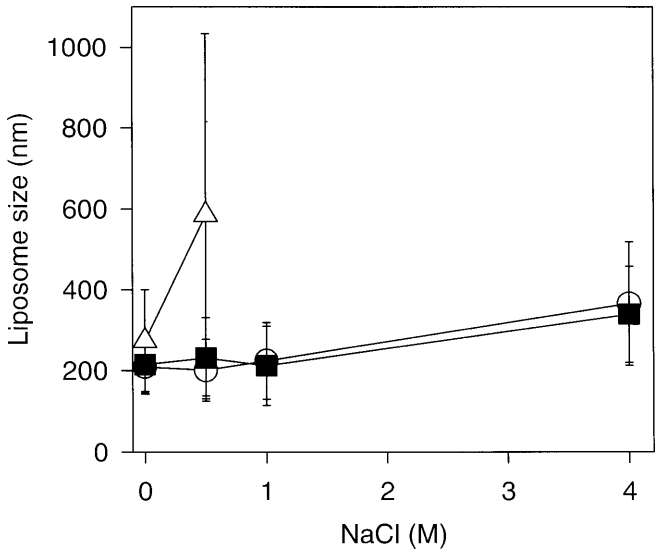

Fig. 1. Influence of NaCl concentration on size of liposomes prepared from *E. coli* (*triangles*), *Halorubrum vacuolatum* (*circles*), and *Halobacterium salinarum* (*squares*) lipids. Standard error of the mean ($n = 3$) is indicated by error bars

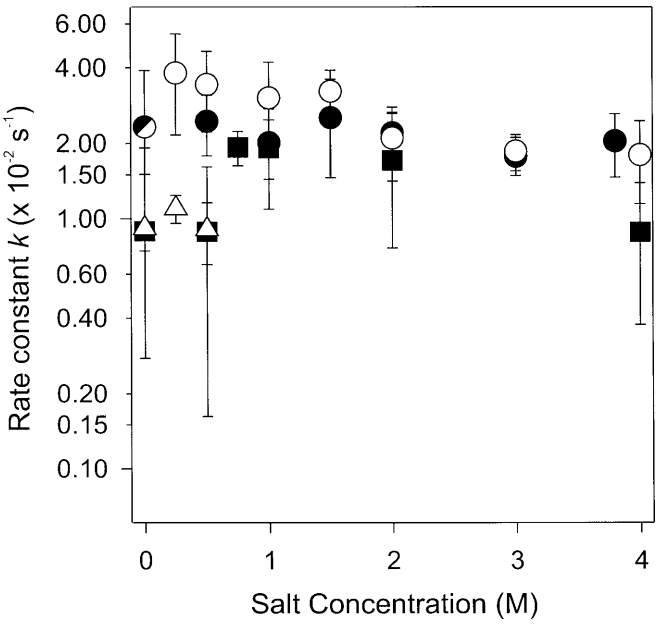

Fig. 2. Influence of NaCl concentration on proton permeability of liposomes prepared from *E. coli* (*triangles*), *Hr. vacuolatum* (*open circles*), and *Hb. salinarum* (*squares*) lipids. Effect of KCl (*solid circles*) concentration on the proton permeability of liposomes prepared from *Hr. vacuolatum* lipids. Standard error of the mean ($n = 3$) is indicated by error bars

vacuolatum. *E. coli* bacterial lipids were used for comparison. All species were grown around 35°C, both Archaea at high salt concentration, *Hr. vacuolatum* at high pH and both neutrophils (*E. coli* and *Hb. salinarum*) at pH 7. Liposomes prepared at various salt concentrations were made from extracted lipids by hydration followed by freeze-thawing and extrusion through 200-nm polycarbonate filters. The size distribution of the liposomes was determined by photon correlation spectroscopy. At low salt concentration, the mean diameter of all liposome preparations was found to be about 200 ± 70 nm (Fig. 1). At 0.5 M NaCl, the *E. coli* liposomes aggregated, resulting in an apparent dramatic size increase to about 550 nm with a large size distribution. At even higher NaCl concentration, the *E. coli* liposomes aggregated even more extensively and started to float. In contrast, the size of liposomes prepared from the lipids from *Hr. vacuolatum* and *Hb. salinarum* increased from 200 to 300 nm with the increase from 0 to 4 M NaCl (Fig. 1). The size of all liposome preparations (including *E. coli*) was unaffected by the pH applied during liposome preparation between pH 7 and 9 (data not shown). The liposomes prepared from *Hr. vacuolatum* and *Hb. salinarum* lipids were stable over a wide range of NaCl concentrations (i.e., to 4 M) and retained the ability to maintain a $\Delta\psi$, applied by a valinomycin-induced potassium diffusion gradient, even after the 1-week incubation at 20°C.

Salt dependency of the proton permeability

The proton permeability of liposomes, derived from *Hr. vacuolatum* and *Hb. salinarum*, was tested at 38°C at various NaCl or KCl concentrations with the acid-pulse method, using the fluorescent dye pyranine to monitor changes in extracellular pH. At low sodium ion concentration, below 0.5 M NaCl, the proton permeability of liposomes from *Hb. salinarum* lipids was equal to that of liposomes from *E. coli* lipids (Fig. 2), whereas liposomes from *Hr. vacuolatum* lipids were about twice as permeable to protons. The proton permeability of liposomes from *Hr. vacuolatum* and *Hb. salinarum* lipids decreased about two-fold at higher NaCl concentration (Fig. 2). Similar observations were made when NaCl was replaced with KCl (Fig. 2). Because of the aggregation of the liposomes from *E. coli* lipids, reliable measurements of the proton permeability at NaCl concentrations above 0.5 M was impossible. The data show that the proton permeability of the liposomes prepared from lipids extracted from the halopilic Archaea is only affected to a minor extent by the salt concentration.

pH dependency of proton permeability

Hr. vacuolatum is a haloalkaliphile that grows optimally between pH 8.5 and 10.5, while *Hb. salinarum* grows below pH 8.5. The proton permeabilities of the liposomes, prepared from lipids of these organisms and of *E. coli*, were measured as a function of pH at a NaCl concentration of 0.1 M. The proton permeabilities of all liposomes between pH 7 and 9 varied only marginally with pH (Fig. 3). The permeabilities of both archaeal liposome preparations were somewhat higher than in liposomes of *E. coli* lipids.

Salt dependency of sodium ion permeability

The sodium ion permeability of liposomes was measured from the efflux of ^{22}Na, preloaded into the liposomal lumen. For liposomes from *Hb. salinarum* lipids, and to a lesser

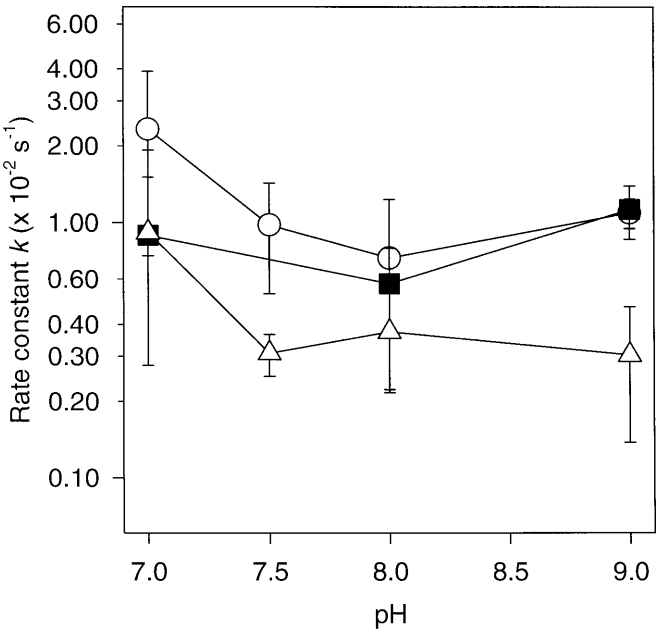

Fig. 3. pH dependency of proton permeability of liposomes prepared from *E. coli* (*triangles*), *Hr. vacuolatum* (*circles*), and *Hb. salinarum* (*squares*) lipids. Standard error of the mean ($n = 3$) is indicated by error bars

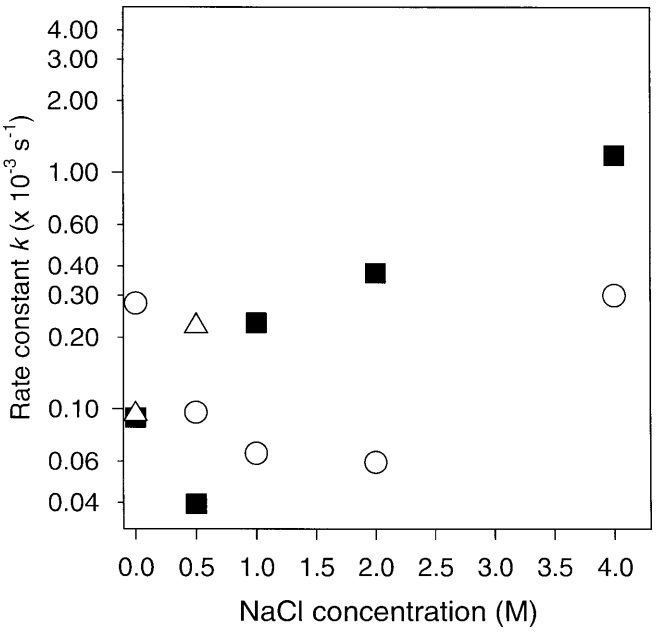

Fig. 4. Influence of NaCl concentration on sodium ion permeability of liposomes prepared from *E. coli* (*triangles*), *Hr. vacuolatum* (*circles*), and *Hb. salinarum* (*squares*) lipids

extent for liposomes from *Hr. vacuolatum* lipids, the first-order rate constant for the sodium ion permeability increased with increasing NaCl concentrations from 0.1 to 4 M (Fig. 4). The Na^+ permeability is about two orders of magnitude lower than the proton permeability at all salt concentrations. The instability of the liposomes from *E. coli* lipids in a highly salted medium prevented the study of these liposomes at high NaCl concentrations. At low NaCl concentration, the permeability of liposomes from *E. coli* lipids was comparable with both liposome preparations derived from both halobacterial lipids.

Discussion

The results reported here show that stable liposomes can be prepared from the lipids derived from halophilic Archaea. Liposomes of lipids from *E. coli* do not remain intact at high salt concentrations as the result of extensive aggregation. Liposomes prepared from lipids derived from the halophile and haloalkaliphile behave similarly on increasing salt concentration. At increasing salt concentration, the size of these liposomes increases slightly while the proton permeability remains constant. A moderate increase in the first-order rate constant for sodium ion permeation was observed with increasing salt concentration. The proton permeability of the haloalkaliphilic liposomes from *Hr. vacuolatum* lipids was not affected by the pH of the medium, and was within the same range as previously observed for other organisms grown around 35°C (Elferink et al. 1994; Van de Vossenberg et al. 1995). The proton permeability is, however, much (more affected by the temperature, i.e., 10 fold over a temperature range of about 30°C (Van de Vossenberg et al. 1995).

The lipid composition of membranes from only a few halo(alkali)philic Archaea has been analyzed (Upasani et al. 1994; Kates 1996). The lipid species present in the phylogenetically closely related *Hr. vacuolatum* and *Hb. salinarum* membranes are very similar (Mwatha and Grant 1993). *Halobacteria* have a high density of negative charges on the surface of their membranes (Russell 1989). The advantage would be that the negative charges on the polar headgroups are shielded by the high ionic concentration, preventing disruption of the lipid bilayers due to charge-repulsive forces and providing a charge-stabilized lipid bilayer (Kates 1993).

Yamauchi et al. (1992) compared the stability of *Halobacteria*-like (Archaea-like) lipids with phospholipids that resemble typical bacterial lipids. They only studied lipids that differed in the acyl chains, using 1,2-diphytanyl-*sn*-glycero-3-phosphocholine as the archaeal model and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine as the bacterial counterpart. The phytanyl (Archaea-like) lipids were found to be stable and could form a concentrated suspension of liposomes at a wide range of salt concentrations. In contrast, the palmitoyl (bacteria-like) lipids could only form liposomes at the higher salt concentrations when prepared at very low lipid concentrations. The phytanyl lipids were found to be less permeable for carboxyfluorescein than the palmitoyl lipids (Yamauchi et al. 1992). The high stability of the halo(alkali)phile phytanyl chain may result from the limited segmentary motion of tertiary carbon atoms (i.e., rotation of carbon atoms that are bound to three other C-atoms) that prevents kink formation in the archaeal phytanyl chains (Degani et al. 1980). This restriction in acyl

chain mobility may enhance the stability of the halophilic lipids at high salt concentration and keep the permeability low. The phytanyl chain is an important determinant for the exceptional properties of the halobacterial lipids.

Halophilic lipids are known to resist high pH values (Kates 1995). It is, therefore, not surprising that liposomes from *Hb. salinarum* and *Hr. vacuolatum* lipids behave similarly under the conditions studied. In contrast to moderate halophiles, which are mostly bacteria, the extreme halophiles do not adapt their membrane lipid composition to fluctuations in salt concentrations. Moderate halophiles increase the amount of negatively charged lipids upon increasing the salt concentration (Russell 1989). Our data suggest that the membranes of halophiles and haloalkaliphiles are mainly adjusted to the high salt concentration and to a lesser extent to pH. Irrespective of the nature of the lipids, i.e., halophile versus haloalkaliphile, pH does not interfere with the membrane integrity, nor does it dramatically affect the proton permeability. In contrast, liposomes of membrane lipids of the acidophilic archaeon *Picrophilus oshimae*, which thrives at pH 1 and 60°C, are unstable at pH values above 5 and collapse at the higher pH values (Van de Vossenberg et al. 1998b). Also, the proton permeability of liposomes prepared from *Sulfolobus acidocaldarius* lipids was found to increase when pH increased from 4 to 7.

At moderate salt concentration, the Na^+ conductance of the liposomes from archaeal lipids is similar to that found previously for bacterial liposomes (Van de Vossenberg et al. 1995). The sodium ion permeability moderately increases with the salt concentration. Consequently, we conclude that the use of sodium ions as coupling ions does not seem to be more advantageous for halophiles than for nonhalophiles under the same conditions. Because halophiles face a large Na^+ gradient, sodium ion-coupled transport can be useful. It can be concluded that neither the sodium ion permeability nor the proton permeability characteristics of halophilic Archaea are significantly different from organisms that live at the same temperature, albeit at low salt concentrations. The main adaptation of halo(alkali)philic membranes is their stability at high salt concentrations.

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