Available online at www.sciencedirect.com

ScienceDirect

Biochimica et Biophysica Acta 1768 (2007) 2157–2163

Biochimica et Biophysica Acta BBA

www.elsevier.com/locate/bbamem

Glycocardiolipin modulates the surface interaction of the proton pumped by bacteriorhodopsin in purple membrane preparations

Angela Corcelli [a], Simona Lobasso [a,*], Matilde Sublimi Saponetti [a], Andreas Leopold [b], Norbert A. Dencher [b]

[a] *Dipartimento di Biochimica medica, biologia medica e fisica medica. Università degli Studi di Bari, Piazza G. Cesare, I-70124 Bari, Italy*

[b] *Physical Biochemistry, Department of Chemistry, Darmstadt University of Technology, Petersenstr. 22, D-64287 Darmstadt, Germany*

Received 7 December 2006; received in revised form 29 June 2007; accepted 29 June 2007
Available online 7 July 2007

Abstract

Glycocardiolipin is an archaeal analogue of mitochondrial cardiolipin, having an extraordinary affinity for bacteriorhodopsin, the photoactivated proton pump in the purple membrane of *Halobacterium salinarum*. Here purple membranes have been isolated by osmotic shock from either cells or envelopes of *Hbt. salinarum*. We show that purple membranes isolated from envelopes have a lower content of glycocardiolipin than standard purple membranes isolated from cells. The properties of bacteriorhodopsin in the two different purple membrane preparations are compared; although some differences in the absorption spectrum and the kinetic of the dark adaptation process are present, the reduction of native membrane glycocardiolipin content does not significantly affect the photocycle (M-intermediate rise and decay) as well as proton pumping of bacteriorhodopsin. However, interaction of the pumped proton with the membrane surface and its equilibration with the aqueous bulk phase are altered.

Keywords: Archaeal cardiolipin; Bacteriorhodopsin; Proton pumping; Photocycle; Proton transfer

1. Introduction

Recently cardiolipin analogues have been discovered in the membrane of archaeal microorganisms of the *Halobacteriaceae* family living in hypersaline environments [1].

As the mitochondrial cardiolipin (CL), the archaeal cardiolipins are dimeric phospholipids having a bridge-like structure; at variance from "authentic" cardiolipin, the hydrophobic tail is not constituted of fatty acids but of phytanyl chains, which are linked to glycerol through ether bonds. Besides an archaeal bisphosphatidylglycerol, other three different cardiolipin analogues have been characterized in the archaeal microorganims, all of them carrying glycosyl moieties in their polar head groups [1–3].

Archaeal cardiolipins, as well as eukaryotic and bacterial cardiolipins, appear to be specifically associated to membrane protein complexes involved in the bioenergetic processes of the cell [1,4–6].

In the model organism *Halobacterium salinarum*, the purple membrane (PM) serves bioenergetic functions, which are driven by redox reactions or chlorophyll-based photosynthesis in other kinds of membranes. The PM contains bacteriorhodopsin (BR), which is a light-driven proton pump transferring one proton across the membrane for each absorbed photon [7].

Two cardiolipin species have been found in the lipid extract of the purple membranes of *H. salinarum*: the archaeal bisphosphatidylglycerol (BPG) and a glycosyl analogue of cardiolipin, named glycocardiolipin (GlyC). While BPG is only a minor lipid component and might be just a contaminant, glycocardiolipin represents an abundant lipid constituent of isolated purple membranes and it has been only found in association with bacteriorhodopsin [4,8]. In standard PM preparation glycocardiolipin represents 25% of membrane lipids; NMR analyses of the PM lipid extract have shown that the GlyC/BR ratio stoichiometry is close to 1 [9].

* Corresponding author. Tel.: +39 80 5448530; fax: +39 80 5448538.
E-mail address: s.lobasso@biologia.uniba.it (S. Lobasso).

0005-2736/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2007.06.029

Interestingly, glycocardiolipin consists of a sulphated-triglycosyl-diphytanylglycerol esterified to the phosphate group of the phosphatidic acid (PA); the sulphated triglycosyldiphytanylglycerol moiety exactly corresponds to the major glycolipid, i.e. S-TGD-1, present in the PM [10]. The glycocardiolipin has been therefore named S-TGD-1-PA (see structure in Fig. 1) [1].

The archaeal cardiolipins are non-abundant or absent in physiological conditions in the halobacterial cells, whereas they are de novo synthesized in the membrane during cell swelling or osmotic shock. It has been reported that the archaeal cells may contain different levels of cardiolipin depending on the extent of osmotic stress [3,11].

In the present study we have been able to isolate purple membranes having lower glycocardiolipin content compared to standard PM.

Previous solubilization and reconstitution studies have indicated that the removal of tightly bound glycocardiolipin destabilizes the BR trimer arrangement and modifies its properties in the presence of detergents [12,13].

The availability of the modified purple membrane isolation procedure affecting the glycocardiolipin content allowed us to probe for the first time the effects of reduced level of this lipid in isolated membrane preparations on BR properties and functions.

The experimental approach illustrated in the present study offers the advantage of selectively changing the amount of just one lipid component of the BR annulus, of avoiding artifacts introduced by the interaction of detergents with membrane components and of reducing the length of the experimental procedures.

2. Materials and methods

2.1. Materials

DNase I was obtained from Sigma. All organic solvents used were commercially distilled and of the highest available purity (Sigma-Aldrich). TLC plates (Silica gel 60 A, 20×10 cm, layer thickness 0.2 mm), obtained from Merck, were washed twice with chloroform/methanol (1:1, v/v) and activated at 120 °C before use.

2.2. Growth conditions

The engineered high-producing BR strain of *Halobacterium salinarum*, used in this study, was kindly provided by Richard Needleman [14]. The *Hbt. salinarum* cells were grown in light at 37 °C in liquid growth medium containing neutralized peptone (L34, Oxoid), prepared as previously described [15].

2.3. Isolation of cell envelopes

The technique for the isolation of cell envelopes is based on a procedure described by Oesterhelt and Stoeckenius [15]. Cells from 7-l culture were resuspended with 200 ml of 4 M NaCl in a plastic container and frozen in liquid N_2. After the liquid nitrogen was evaporated and the cell suspension reached room temperature, 3-4 mg of DNase were added and the gelatinous preparation is stirred for 2 h to decrease viscosity for subsequent centrifugations. The suspension was then diluted fivefold with 4 M NaCl and centrifuged at 4300×*g* for 20 min to remove unbroken cells and large debris; then the supernatant (SN) was centrifuged at 27,000×*g* for 1 h to recover the purple cell envelopes. The envelopes (E) were washed with equal volumes at 4 M NaCl and then suspended in the same solution to 10–20 mg/ml.

2.4. Osmotic shock of cell envelopes

Cell envelopes suspended in 4 M NaCl were dialyzed against H_2O at 4 °C for different incubation times (2, 4 and 12 h). Alternatively, an aliquot of cell envelopes (in 4 M NaCl) was dialyzed for 2 h against 1 M NaCl and then the cell membranes were recovered as below described.

2.5. Purple membrane isolation

Standard purple membranes (PM) were isolated from the high-producing BR cells as previously described [15]. Purple membranes having lower glycocardiolipin content were obtained by dialysis over night of cell envelopes in 4 M NaCl against H_2O at 4 °C. The membranes (PM_E) were sedimented at 27,000×*g* for 1 h and then suspended in water.

Both the PM and PM_E (re-suspended in distilled water) were layered over a step gradient (60%, 35% and 15% sucrose) and then centrifuged 100,000×*g* for 18 h at 10 °C. The purple bands were collected and sucrose removed by dialysis. Finally, the collected PM and PM_E were suspended in water and frozen (−20 °C).

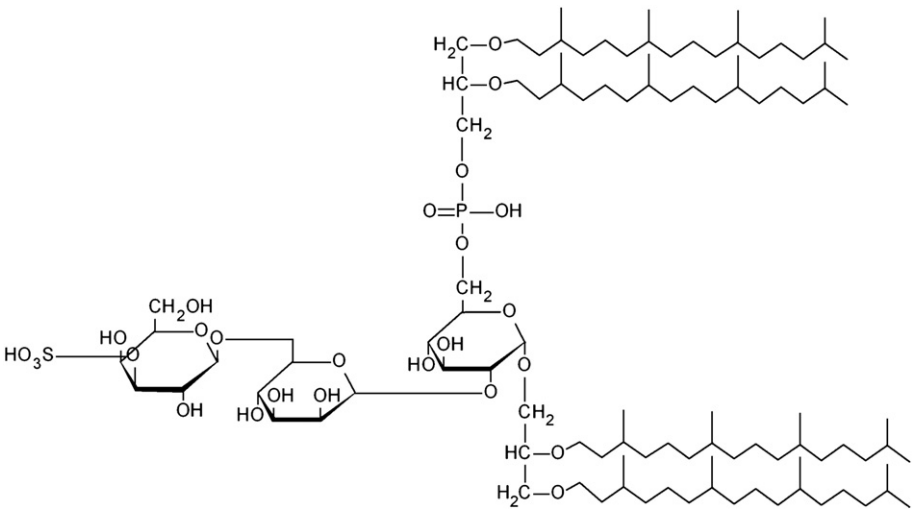

Fig. 1. Structure of glycocardiolipin (GlyC or S-TGD-1-PA).

2.6. Thin-layer chromatography of lipids

Total lipids were extracted using the Bligh and Dyer method, as modified for extreme halophiles [16]. Total lipid extracts were analyzed by TLC with Solvent A (chloroform/methanol/90% acetic acid, 65:4:35, v/v).

Lipid detection was carried out by spraying with 5% sulfuric acid in water, followed by charring at 120 °C for 45 min.

2.7. Quantification of individual lipids in the total lipid extracts

The signal from the chromatogram fraction has been compared to the signal from the sample free plate background. For quantitative determination, peak data of the unknowns are correlated with data from calibration standards chromatographed on the same plate. In the experimental condition used in the present study, the intensity of the staining of the authentic standard lipid raises linearly in the concentration range 1–10 micrograms.

As archaeal lipids are not commercially available, we have extracted, isolated and purified phospholipids from cultures of halobacterial cells. The isolation of individual lipid components of the total extracts was performed by scraping the silica gel in each band from the plate and extracting each band lipids from the silica as previously described [1]. The quantitative analysis was performed by video densitometry, using the software ImageJ (http://rsb.info.nih.gov/ij).

2.8. Absorption spectroscopy

UV-visible absorption spectra were obtained with a Cary 50 UV-visible spectrophotometer. Dark-adapted (DA) spectra were obtained on samples stored in complete darkness for 12 h at room temperature. Light adaptation (LA) of samples was accomplished using a 250-W illuminator. A cuvette (1 cm path length) containing 9 μM bacteriorhodopsin in water was irradiated at a distance of 15 cm from the bulb for 5 min. The rate of dark adaptation of bacteriorhodopsin was measured by following at room temperature absorption decrease at the wavelength in the visible region with the largest absorption difference between LA and DA forms, in particular at 585 and 590 nm for PM and PM_E, respectively.

2.9. M-Intermediate kinetics

To initiate the photocycle of bacteriorhodopsin, the sample was irradiated by a pulsed-laser at $\lambda = 532$ nm (8 ns duration) and we monitored the transient absorbance changes at 412 nm and 568 nm, characteristic wavelengths for the M-intermediate and the ground-state, respectively. The average laser energy was about 8–12 mJ, which resulted in an irradiation of 2–3 mJ/cm^2 on the cuvette. The transient absorption changes were analyzed according to ref [17].

Time-resolved changes in absorbance at selected wavelengths were digitized with a transient recorder (eight different sampling rates, 100 MHz maximum sampling rate, 10 bit resolution, 524288 data points memory). This set up enabled to detect transients over at least 8 decades in time (65536 data points each) with a resolution of 20 ns. The high density of sampling points leads to a high signal-to-noise ratio when neighboring points are co-added. Further improvement of the signal-to-noise ratio is achieved by averaging 40 (BR photocycle) data traces.

Fitting of the weighted data points to a sum of exponentials was done with a program that uses the Marquardt–Levenberg algorithm. The fit was accepted when the residuals were free from systematic deviations. The original data traces were fitted with a sum of five exponentials, three for the formation and two for the decay of the M-intermediate. However, since this leads to five rate constants and five amplitude values that vary for the various samples, for direct comparison the time-course of the rise and decay of the signals was described only with a single exponential each. This had no significant effect on the interpretation of the data in terms of the efficiency of BR as a light-driven proton pump.

2.10. Bacteriorhodopsin proton pumping

To investigate the transfer of protons liberated from BR into the aqueous bulk phase, pyranine (8-oxypyrenetrisulphonic acid) was employed as indicator [18]; the transient absorbance changes of the pH-indicator pyranine were adequately fitted by two exponentials, as also had been demonstrated in refs. [17–19].

The experiments were carried out in 150 mM KCl and the pH was adjusted to 6.90±0.02 before each measurement by HCl and NaOH, respectively, from 1 M to 1 mM stock solutions. The purple membrane suspensions had a final BR concentration of 0.25 mg/ml.

3. Results

Two different purple membrane preparations have been compared in the present study. By following the method of Oesterhelt and Stoeckenius [15], which represents the standard

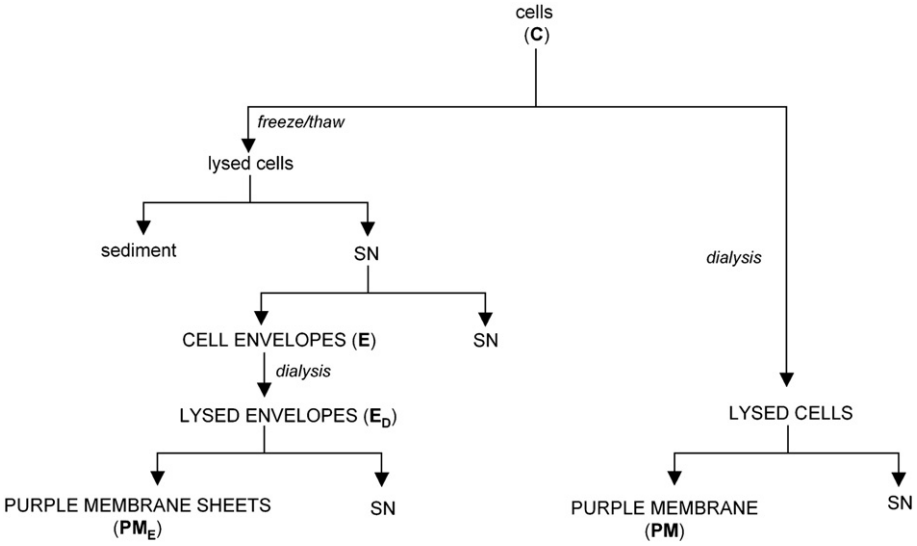

Fig. 2. Scheme of isolation of purple membranes sheets from cell envelopes of *Halobacterium salinarum*. Cells suspended in 4 M NaCl were frozen in a plastic container in liquid N_2. After the liquid nitrogen was evaporated and the cell suspension reached room temperature, 3–4 mg of DNase was added and the gelatinous preparation is stirred for 2 h. After dilution with 4 M NaCl, the suspension was centrifuged at 4,300×*g* for 20 min and the supernatant (SN) was then centrifuged at 27,000×*g* for 1 h to recover the purple cell envelopes (E). Purple membranes sheets (PM_E) were obtained by dialysis over night of cell envelopes in 4 M NaCl against H_2O at 4 °C. The PM_E were collected by centrifugation at 27,000×*g* for 1 h and then re-suspended in water.

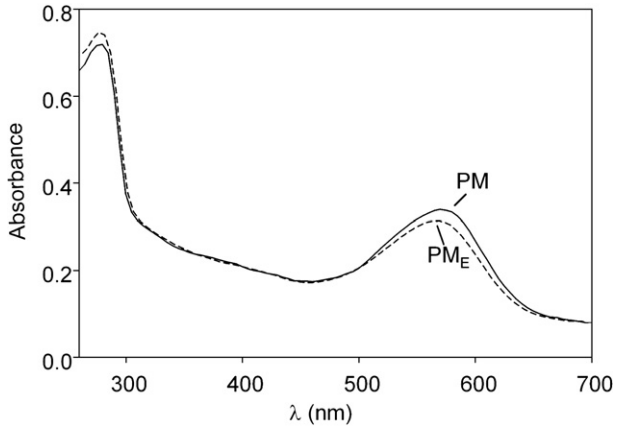

Fig. 3. Absorption spectra of dark-adapted purple membrane (PM) and purple membrane sheets (PM_E). The spectra were obtained after membrane purification on sucrose gradient. Both the samples (in water) were stored in the dark for at least 12 h at room temperature.

procedure followed in most laboratories, purple membranes have been isolated from *Hbt. salinarum* cells lysed in a dialysis bag kept in distilled water. The lipid composition of this purple membrane preparation (named in laboratory jargon PM) has been previously analyzed both with TLC and NMR spectroscopy [1,9,11]; it has been shown that cardiolipins are present in significant amount in this membrane preparation and in particular that the glycocardiolipin/bacteriorhodopsin molar ratio is about 1 [9].

It was previously shown that purple membranes could also be isolated from cell envelopes of *Hbt. salinarum* exposed to low salt containing medium [20]. We have taken advantage of these previous observations and developed a modification of the above method to isolate cell envelopes and purple membrane sheets after dialysis of envelopes (named PM_E in the following). The scheme in Fig. 2 illustrates in details the isolation protocol of purple membrane sheets from cell envelopes. Unless otherwise specified, the pellet containing purple membrane sheets was loaded over a sucrose gradient to purify the membrane. The comparison of the UV-visible absorption spectra of PM and PM_E is reported in Fig. 3: it can be seen that the 280 nm/560 nm absorption ratio, an index of BR purification, of the two spectra was nearly the same.

Fig. 4A shows the TLC of the lipid extracts of *Hbt. salinarum* whole cells (C) and cell envelopes (E), of lysed cell envelopes after dialysis against water (E_D), of PM_E recovered after envelopes dialysis, and of PM isolated by the standard procedure [15].

The individual lipid components of the PM lipid extract were (in R_f order) sulphated tetraglycosyl diphytanylglycerol (S-TeGD-1), sulphated triglycosyl diphytanylglycerol (S-TGD-1), glycocardiolipin (GlyC), phosphatidylglycerosulphate (PGS), phosphatidylglycerophosphate methyl ester (PGP-Me), phosphatidylglycerol (PG) and bisphosphatidylglycerol (BPG), plus pigments and other neutral lipids at the solvent front.

It can be clearly seen that GlyC was practically absent among lipids extracted from both C and E, whereas it was present in E_D, PM, and PM_E. Taken all data together, Fig. 4A suggests that GlyC is formed only after the envelopes as well as the cells are exposed to hypotonic stress, in agreement with our previous studies [11]. Densitometric analysis of TLC in Fig. 4A showed that glycocardiolipin content in PM_E was about 4-fold lower than in standard PM. To determine the time-course of the GlyC neo-synthesis during cell envelopes dialysis, we have extracted lipids from equivalent envelopes aliquots, taken at different times from the starting of dialysis against water and quantified the GlyC content (Fig. 4B). Moreover, the GlyC levels of cell envelopes dialysed against water were found to be higher than that of envelopes dialysed against 1.0 M NaCl for the same time (provided as supplementary data). These data suggest that the GlyC synthesis in the cell envelopes depends on the duration of dialysis and the magnitude of the hypotonic gradient across the envelopes.

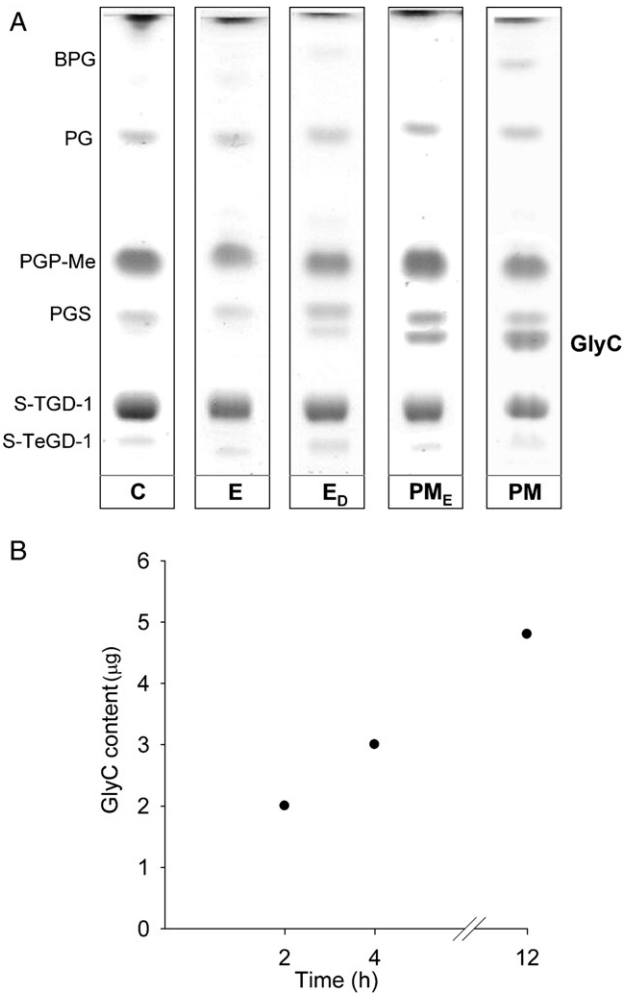

Fig. 4. (A) Thin-layer chromatography (TLC) of the lipid extracts of whole cells (C), cell envelopes (E), lysed cell envelopes after dialysis over night against water (E_D), purple membranes sheets recovered after envelopes dialysis (PM_E) and purple membrane (PM); 60 μg of lipid extracts has been loaded onto each line. The abbreviated names of individual lipid components in the extract have been reported. (B) Time-course of the GlyC increase during cell envelopes dialysis. Cell envelopes suspended in 4 M NaCl were dialyzed against H_2O at 4 °C for different incubation times (2, 4 and 12 h). The GlyC content at the different incubation times has been estimated by video densitometric analysis of TLC, in which 100 μg of each lipid extract has been loaded (as described in details in Materials and methods).

As it has been reported that the neutral lipid squalene may play a role in the stability and function of bacteriorhodopsin [21], the lipid extracts of the two different membrane preparations were also analyzed by TLC with a solvent system specific for the neutral lipids; however no differences in the squalene content of the two extracts could be detected (data not shown).

From the data shown in Figs. 3 and 4, we can conclude that purple membrane fragments (i.e. PM_E) isolated from cell envelopes of *Hbt. salinarum* contain only bacteriorhodopsin as protein and have a GlyC/BR molar ratio of about 1:4; i.e., PM_E differs from PM isolated from the whole cells in the reduced glycocardiolipin content only.

We compared the light-dependent properties and proton transfer of bacteriorhodopsin in the two different purple membrane preparations.

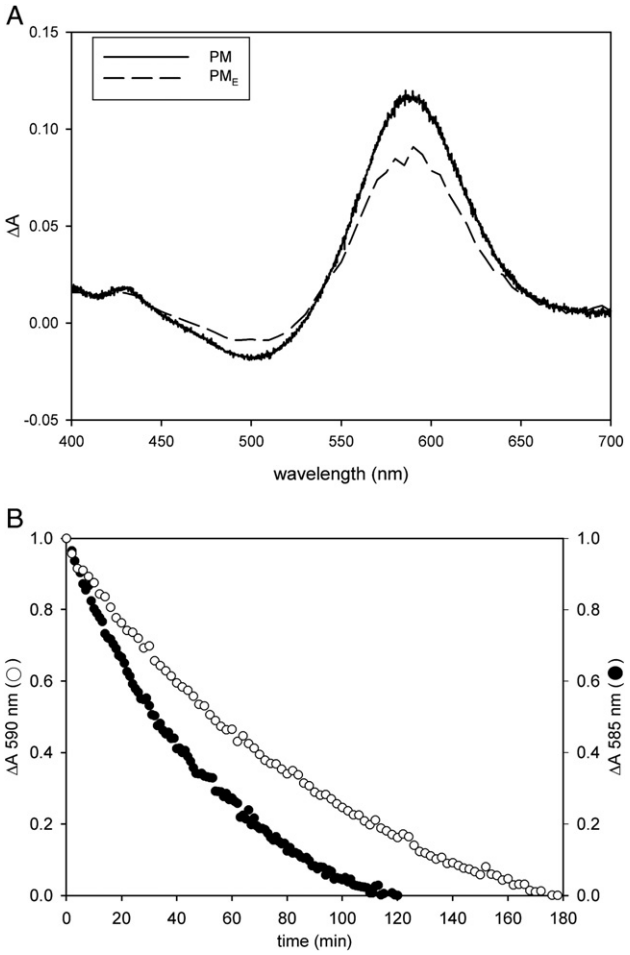

Fig. 5. (A) Light–dark difference spectra of PM (full line) and PM_E (dotted line). Dark-adapted (DA) spectra were obtained on samples stored in complete darkness for 12 h at room temperature. Light adaptation (LA) of samples was accomplished using a 250 W illuminator; a cuvette (1 cm path length) containing 9 μM bacteriorhodopsin in water was irradiated at a distance of 15 cm from the bulb for 5 min. (B) Kinetics of dark-adaptation of PM_E (○) and PM (●). The rate of dark adaptation of bacteriorhodopsin was measured by following at room temperature absorption decrease at the wavelength in the visible region with the largest absorption difference between LA and DA forms; the relative absorbances are reported. The two traces (representative of a typical experiment) were best fitted by single exponentials. The lifetimes were 53.88 min for PM_E (Rsqr 0.9991) and 32.60 for PM (Rsqr 0.9988).

Table 1
Time constants of formation and decay of the M-Intermediate as well as proton release and reuptake monitored by pyranine at 22 °C in 150 mM KCl

	$\tau_{M\ rise}$ [μs]	$\tau_{M\ decay}$ [ms]	τ_{P1} [ms]	τ_{P2} [ms]
PM				
A*	86.6	4.80	1.35	10.30
B*	94.5	4.93	1.20	9.96
C*	94.5	5.23	1.47	11.20
PM_E				
D*	86.6	5.69	0.95	10.75
E*	99.2	5.11	0.91	7.25
F*	97.6	5.05	1.29	9.37

A, B, C, D, E, and F represent different samples of different membrane preparations, and * indicates membranes purified on sucrose gradient. For direct comparison of the various samples, the time-course of the rise and decay of the signals was fitted only with a single exponential each.

For dark-adapted samples, the absorption maximum of PM was at 560 nm, whereas the absorption maximum of PM_E was red-shifted at about 565 nm; no differences have been found in the light-adapted spectra of the two membrane preparations (λ_{max} = 570 nm).

The light–dark difference spectra of PM and PM_E are compared in Fig. 5A, while Fig. 5B shows the kinetics of dark-adaptation of PM and PM_E. Upon the light-adaptation, the return to the dark-adapted state of PM_E was found to be slower than PM; both traces were well fitted by single exponentials, with lifetimes of respectively 53.88 and 32.60 min. Data and statistics for the fittings of both the curves are provided in the figure legend.

To compare the bacteriorhodopsin photocycle in PM and PM_E, the kinetics for M-intermediate rise and decay were determined. Table 1 (first and second column) depicts the time constants of the rise and decay of M-Intermediate of PM (samples A, B and C) and PM_E (samples D, E and F). The rise and decay of the M-intermediate were quite similar for all samples. For the results obtained at 22 °C, less GlyC (PM_E) correlated with only about 2.8% and 6% larger rise and decay time-constants respectively, in comparison to the normal content (PM). If the data obtained at all investigated temperatures (10, 22, 28, 35, and 55 °C) are considered (not shown) the slight differences in the photocycle kinetics observed were not significant.

Furthermore, light-induced proton translocation by bacteriorhodopsin residing in PM and PM_E has been investigated by using the optical pH-pyranine. In contrast to the photocycle kinetics reflecting the internal proton translocation steps [18,19], the time constants of the pyranine signal were significantly smaller for PM_E with reduced GlyC content. The rate of protonation of pyranine (τ_{P1}) in the aqueous bulk phase and its subsequent deprotonation (τ_{P2}) at 22 °C were accelerated by about 25% and 13%, respectively, as compared to PM samples (Table 1, third and fourth column). From the measurements at various temperatures it can be concluded that it is especially the proton transfer from the membrane surface into the aqueous medium (indicated by τ_{P1}, [17–19]) that is facilitated at decreased GlyC content. However, it has to be mentioned that the effect of GlyC on the pyranine signals is smaller than that caused by membrane aggregation, for example.

4. Discussion

The archaeal microorganism *Halobacterium salinarum* inhabits the hypersaline red water of salt-ponds and requires 4 M NaCl to grow regularly in culture media. Studies of cell lysis in media with low salt concentration indicated that the reduction of the salt concentration caused the release of the cell wall before the cell membranes begin to disintegrate [20]. As a consequence of osmotic shock only some membrane fragments can be collected by centrifugation; most of these membrane fragments are constituted of purple membranes, which does not require salt for stabilization.

The effect of lowering the salt concentration on the wall of Halobacteria was also studied in detail in cell envelopes isolated from *Hbt. salinarum* by freeze–thaw [20]; these morphological studies indicated that in the envelopes, as well as in the intact cells, the first visible step after the lysis was a loss of cell wall material; below 1.0 M NaCl the vesicles opened and the cell membranes disintegrated, so that less and less material remained sedimentable. A residue of membranous purple colored sheets remained even after prolonged exposure of halobacterial envelopes to distilled water.

Our biochemical study adds novel information on the composition and response to osmotic stress of membrane vesicles or envelopes isolated from *Hbt. salinarum* cells.

We show that glycocardiolipin appears to be absent in the lipid extract of cell envelopes of *Hbt. salinarum*. Only after incubation of envelopes in hyposmotic media (Fig. 4A), glycocardiolipin has been found in the envelope lipid extract. The levels of glycocardiolipin in the membrane envelopes increase by increasing the incubation time (Fig. 4B) and depend on the extent of osmotic unbalance between the solutions outside and inside the envelopes.

Likely due to loss or impairment of some membrane components during the envelope isolation, the neo-synthesis of glycocardiolipin in the vesicles is less efficient than that occurring in the intact cells; for this reason the PM_E isolated from envelopes contains a reduced glycocardiolipin level in the membrane matrix (Fig. 4A).

Therefore our study illustrates the first case of content modulation of a specific lipid component during isolation of native membrane domains; the two different purple membranes represent a useful model to check if reduced cardiolipin level in native membrane may affect the bioenergetic function of the membrane.

In fact, the literature reports a number of studies showing that the reduction of CL levels compromises directly the function of several essential enzymes and processes of the mitochondria (for reviews see [5,22–24]); most of these reports refer to solubilization and purification studies, but also genetic studies on CL lacking mutants are available [25–27].

Here we report significant kinetic differences in the dark-adaptation process after continuous illuminations of PM and PM_E (Fig. 5A and B), suggesting a specific role for the lipid–protein interaction in the process of dark-adaptation of PM, as previously shown for other lipids [28]. On the other hand, the photocycle and the accompanying of internal proton transfer steps across the membrane do not differ between the samples with regular and reduced GlyC-content.

What is modulated by the GlyC content, however, is the interaction of the pumped proton with the membrane surface and therefore its equilibration with the aqueous bulk phase. As has been convincingly demonstrated previously [7,17–19], the pyranine signal τ_{P1} does not monitor the light-triggered proton transfer steps inside BR to the membrane surface, that occur in about 100 μs, but the delayed equilibration of the pumped proton into the aqueous bulk phase. Therefore, the smaller τ_{P1} for PM_E indicates that due to lower/reduced GlyC content the protons are retained at the membrane surface for a shorter time as compared to the "normal" PM, i.e. they are released faster.

To better understand the role of glycocardiolipin in the BR function, it would be interesting also to study mutant microorganisms lacking glycocardiolipin; however either evidence for the presence of an archaeal cardiolipin synthase has not yet been reported nor such a mutant organism is available. Although until now the maximum glycocardiolipin level found in PM in our and other laboratories is that corresponding to a GlyC/BR molar ratio 1:1 [9], it would be useful to find an experimental method to isolate PM with higher GlyC-content than the standard PM. The availability of GlyC-enriched PM would allow testing if GlyC head groups (together with that of the other highly acidic PM phospholipids) might act as reservoir for protons to be pumped across the membrane, in analogy with the model proposed for mitochondrial CL [29].

In conclusion, we suggest that during the exposure to hypotonic medium the cell performs GlyC neo-synthesis, which induces a change in the charge distribution over the membrane surface helping the cell to better redistribute the voltage generated in the purple membrane domains to others, where the ATP synthesis occurs and, therefore, to optimize the cell response to stress.

At the same time, the ability of the cell to increase cardiolipin content [3,11] might represent a safety valve through which, due to the retardation of proton release over the membrane surface, the formation of an excess driving force in stressful situations is avoided.

However, results and data obtained in the present study imply that in normal or non-stress conditions bacteriorhodopsin may function in the absence of GlyC as well and that researchers should take in account that studies of BR properties in isolated PM may be distorted because of the unnatural abundance of GlyC in these preparations.

Acknowledgments

This work was financially supported by grants from Ministero Italiano dell'Università e della Ricerca MIUR (Bari University funds 2006), from the Ministero Italiano della Difesa (contract n. 9199/13.12.05) and from the Istituto per i Processi Chimico-Fisici, Consiglio Nazionale delle Ricerche (IPCF-CNR, Bari unit, Italy) as well as by the Deutsche Forschungsgemeinschaft Forschungs (SFB472 to N.A.D.). We thank Dr. Bettina Schatzler for helpful discussion.

References

[1] A. Corcelli, M. Colella, G. Mascolo, F.P. Fanizzi, M. Kates, A novel glycolipid and phospholipid in the purple membrane, Biochemistry 39 (2000) 3318–3326.

[2] G.D. Sprott, S. Larocque, N. Cadotte, C.J. Dicaire, M. McGee, J.R. Brisson, Novel polar lipids of halophilic eubacterium Planococcus H8 and archaeon *Haloferax volcanii*, Biochim. Biophys. Acta 1633 (2003) 179–188.

[3] P. Lopalco, S. Lobasso, F. Babudri, A. Corcelli, Osmotic shock stimulates de novo synthesis of two cardiolipins in an extreme halophilic archaeon, J. Lipid Res. 45 (2004) 194–201.

[4] A. Corcelli, V.M.T. Lattanzio, A. Oren, The archaeal cardiolipins of the extreme halophiles, in: A. Ventosa (Ed.), Halophilic Microorganisms, Springer-Verlag, Berlin Heidelberg, 2004, pp. 204–214.

[5] E. Mileykovskaya, M. Zhang, W. Dowhan, Cardiolipin in energy transducing membranes, Biochemistry (Moscow) 70 (2005) 191–196.

[6] A. Corcelli, S. Lobasso, Characterization of lipids of halophilic Archaea, in: F.A. Rainey, A. Oren (Eds.), Extremophile microorganisms and the methods to handle them, Methods in Microbiology, vol. 35, Elsevier, Amsterdam, 2006, pp. 585–614.

[7] N.A. Dencher, H.J. Sass, G. Büldt, Water and bacteriorhodopsin: structure, dynamics and function, Biochim. Biophys. Acta 1460 (2000) 192–203.

[8] V.M.T. Lattanzio, A. Corcelli, G. Mascolo, A. Oren, Presence of two novel cardiolipins in the halophilic archaeal community in the crystallizer brines from the salterns of Margherita di Savoia (Italy) and Eilat (Israel), Extremophiles 6 (2002) 437–444.

[9] A. Corcelli, V.M.T. Lattanzio, G. Mascolo, P. Papadia, F.P. Fanizzi, Lipid–protein stoichiometries in a cristalline biological membrane: NMR quantitative analysis of the lipid extract of the purple membrane, J. Lipid Res. 43 (2002) 132–140.

[10] M. Kates, P.W. Deroo, Structure determination of the glycolipid sulfate from the extreme halophile *Halobacterium cutirubrum*, J. Lipid Res. 14 (1973) 438–445.

[11] S. Lobasso, P. Lopalco, V.M.T. Lattanzio, A. Corcelli, Osmotic shock induces the presence of glycocardiolipin in the purple membrane of *Halobacterium salinarum*, J. Lipid Res. 44 (2003) 2120–2126.

[12] F. Lopez, S. Lobasso, M. Colella, A. Agostiano, A. Corcelli, Light-dependent and biochemical properties of two different bands of bacteriorhodopsin isolated on phenyl-sepharose CL-4B, Photochem. Photobiol. 69 (1999) 599–604.

[13] L. Catucci, V.M.T. Lattanzio, S. Lobasso, A. Agostiano, A. Corcelli, Role of endogenous lipids in the chromophore regeneration of bacteriorhodopsin, Bioelectrochemistry 63 (2004) 111–115.

[14] B.F. Ni, M. Chang, A. Duschl, K. Lanyi, R. Needleman, An efficient system for the synthesis of bacteriorhodopsin in *Halobacterium halobium*, Gene 90 (1990) 169–172.

[15] D. Oesterhelt, W. Stoeckenius, Isolation of cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane, Methods Enzymol. 31 (1974) 667–678.

[16] M. Kates, Lipid extraction procedures, in: R.H. Burdon, H. van Knippenberg (Eds.), Techniques of Lipidology, Elsevier, Amsterdam, 1986, pp. 100–111.

[17] J. Heberle, J. Riesle, G. Thiedemann, D. Oesterhelt, N.A. Dencher, Proton migration along the membrane surface and retarded surface to bulk transfer, Nature 370 (1994) 379–382.

[18] S. Grzesiek, N.A. Dencher, Time-course and stoichiometry of H^+-release and uptake during the photochemical cycle of bacteriorhodopsin, FEBS Lett. 208 (1986) 337–342.

[19] J. Heberle, N.A. Dencher, Surface-bound optical probes monitor proton translocation and surface potential changes during the bacteriorhodopsin photocycle, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 5996–6000.

[20] W. Stoeckenius, R. Rowen, A morphological study of *Halobacterium halobium* and its lysis in media of low salt concentration, J. Cell Biol. 34 (1967) 365–393.

[21] R.W. Hendler, S.M. Barnett, S. Dracheva, S. Bose, I.W. Levin, Purple membrane lipid control of bacteriorhodopsin conformational flexibility and photocycle activity, Eur. J. Biochem. 270 (2003) 1920–1925.

[22] F.L. Hoch, Cardiolipins and biomembrane function, Biochim. Biophys. Acta 1113 (1992) 71–133.

[23] M. Schlame, D. Rua, M.L. Greenberg, The biosynthesis and functional role of cardiolipin, Prog. Lipid Res. 39 (2000) 257–288.

[24] W. Dowhan, E. Mileykovskaya, M. Bogdanov, Diversity and versatility of lipid–protein interactions revealed by molecular genetic approaches, Biochim. Biophys. Acta 1666 (2004) 19–39.

[25] D.B. Ostrander, M. Zhang, E. Mileykovskaya, M. Rho, W. Dowhan, Lack of mitochondrial anionic phospholipids causes an inhibition of translation of protein components of the electron transport chain. A yeast genetic model system for the study of anionic phospholipid function in mitochondria, J. Biol. Chem. 276 (2001) 25262–25272.

[26] M. Zhang, E. Mileykovskaya, W. Dowhan, Gluing the respiratory chain together. Cardiolipin is required for supercomplex formation in the inner mitochondrial membrane, J. Biol. Chem. 277 (2002) 43553–43556.

[27] M. Zhang, X. Su, E. Mileykovskaya, A.A. Amoscato, W. Dowhan, Cardiolipin is not required to maintain mitochondrial DNA stability or cell viability for *Saccharomyces cerevisiae* grown at elevated temperatures, J. Biol. Chem. 278 (2003) 35204–35210.

[28] N.A. Dencher, K.D. Kohl, M.P. Heyn, Photochemical cycle and light–dark adaptations of monomeric and aggregated bacteriorhodopsin in various lipid environments, Biochemistry 22 (1983) 1323–1334.

[29] T.H. Haines, N.A. Dencher, Cardiolipin: a proton trap for oxidative phosphorylation, FEBS Lett. 528 (2002) 35–39.