



## Archaeobacterial lipid membranes as models to study the interaction of 10-*N*-nonyl acridine orange with phospholipids

Simona Lobasso<sup>a</sup>, Matilde Sublimi Saponetti<sup>a</sup>, Francesco Polidoro<sup>a</sup>, Patrizia Lopalco<sup>a</sup>, Jasna Urbanija<sup>b</sup>, Veronika Kralj-Iglic<sup>b</sup>, Angela Corcelli<sup>a,\*</sup>

<sup>a</sup> Department of Medical Biochemistry, Medical Biology and Medical Physics, University of Bari, P.zza G. Cesare, I-70124 Bari, Italy

<sup>b</sup> Institute of Biophysics, University of Ljubljana, Ljubljana, Slovenia

### ARTICLE INFO

#### Article history:

Received 9 January 2008

Received in revised form

17 September 2008

Accepted 18 September 2008

Available online 30 September 2008

#### Keywords:

Ether lipids

Phospholipids

Cardiolipin

Archaea

NAO

### ABSTRACT

The dye 10-*N*-nonyl acridine orange (NAO) is used to label cardiolipin domains in mitochondria and bacteria. The present work represents the first study on the binding of NAO with archaeobacterial lipid membranes. By combining absorption and fluorescence spectroscopy with fluorescence microscopy studies, we investigated the interaction of the dye with (a) authentic standards of archaeobacterial cardiolipins, phospholipids and sulfoglycolipids; (b) isolated membranes; (c) living cells of a square-shaped extremely halophilic archaeon. Absorption and fluorescence spectroscopy data indicate that the interaction of NAO with archaeobacterial cardiolipin analogues is similar to that occurring with diacidic phospholipids and sulfoglycolipids, suggesting as molecular determinants for NAO binding to archaeobacterial lipids the presence of two acidic residues or a combination of acidic and carbohydrate residues. In agreement with absorption spectroscopy data, fluorescence data indicate that NAO fluorescence in archaeal membranes cannot be exclusively attributed to bisphosphatidylglycerol and, therefore, different from mitochondria and bacteria, the dye cannot be used as a cardiolipin specific probe in archaeal microorganisms.

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### 1. Introduction

The fluorescent dye 10-*N*-nonyl-3,6-bis(dimethylamino)-acridine (10-*N*-nonyl acridine orange, NAO) is able to penetrate living eukaryotic cells and specifically label the inner mitochondrial membrane (Ratinaud et al., 1988; Lizard et al., 1990; Maftah et al., 1989). NAO is a highly specific stain for negatively charged phospholipids and, more particularly, for cardiolipin (CL or bisphosphatidylglycerol) (Petit et al., 1992, 1994), which is found exclusively in mitochondrial, chloroplast and bacterial membranes (Schlame et al., 2000; Ventrella et al., 2007).

It is generally accepted that NAO binds to anionic phospholipids of the cells owing to an interaction between its quaternary amine and the phosphate residue of phospholipids and an intercalation of the hydrophobic moiety into the membrane bilayer (see structure in Fig. 1) (Petit et al., 1992; Mileykovskaya et al., 2001).

The specificity of NAO binding to cardiolipin has been attributed to the peculiar structural features of this phospholipid. However, the precise mechanism of its selective fluorescence in the presence of CL is not completely understood.

Since it has two phosphate groups on the polar head and four lipid chains in the hydrophobic tail, cardiolipin is unique compared to other acidic phospholipids. It should be considered a dimeric phospholipid, rather than a simple phospholipid.

NAO has been used *in vivo* to identify changes in the distribution of this phospholipid in the mitochondrion during apoptosis (Garcia Fernandez et al., 2000; Nomura et al., 2000; Sorice et al., 2000) and to visualize cardiolipin-rich domains in yeasts (Gallet et al., 1995), *Escherichia coli* (Mileykovskaya and Dowhan, 2000) and *Bacillus subtilis* (Kawai et al., 2004) cells.

As cardiolipin analogues have recently been discovered in the membrane of the archaeal extremophilic microorganisms of the *Halobacteriaceae* family living in hypersaline environments (Corcelli et al., 2000; Lattanzio et al., 2002; Sprott et al., 2003; Lopalco et al., 2004), it is of interest to know whether NAO might also be a useful tool to identify the cardiolipin domains of archaeal microorganisms.

Extremophilic Archaea thrive in hostile conditions, such as salt brines, hot springs, and acidic or alkaline environments. Their membrane lipids differ from those of eukarya and bacteria because they contain exclusively phytanyl chains, which are ether-linked to the C2 and C3 of *sn*-glycerol (archaeol) (Kates, 1993). The major polar head groups on C1 are phosphatidylglycerophosphate methyl ester (PGP-Me), phosphatidylglycerosulfate (PGS), phosphatidylglycerol (PG), and sulfated triglycosyl or diglycosides

\* Corresponding author. Tel.: +39 080 5448530; fax: +39 080 5448538.  
E-mail address: [a.corcelli@biologia.uniba.it](mailto:a.corcelli@biologia.uniba.it) (A. Corcelli).

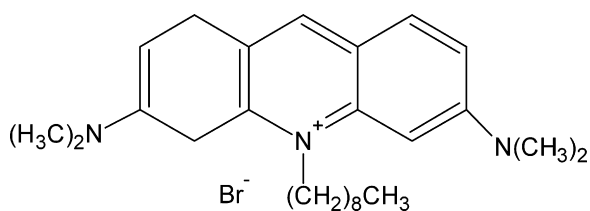


Fig. 1. Structure of 10-N-nonyl acridine orange.

(see structures in Fig. 2). Neither phosphatidylcholine nor phosphatidylethanolamine are present. As all the lipid components are anionic in nature, a high negative density charge is present over the membrane surface, which is balanced by the high sodium and potassium ion concentrations present in the extracellular and intracellular medium, respectively. In this paper the terms archaeal lipids and ether lipids will be used as synonyms to specifically refer to the membrane lipid components of archaeal microorganisms.

Besides bisphosphatidylglycerol (BPG), a number of substituted cardiolipins or analogues have been described in Archaea (Corcelli et al., 2000; Lattanzio et al., 2002; Sprott et al., 2003; Lopalco et al., 2004). Up to now three ether cardiolipin analogues have been described in extreme halophiles (Fig. 3), the first two of them discovered and characterized by our group (Corcelli et al., 2000; Lopalco et al., 2004); these dimeric phospholipids consist of a sulfo-glycolipid (*genus* specific) esterified to phosphatidic acid (PA), and therefore possess two highly acidic groups in their polar heads (a phosphate and a sulfate). As regards the presence and distribution of ether cardiolipin analogues in the extreme halophiles, we have shown that archaeal BPG is generally present (Lattanzio et al., 2002), while each glycosyl substituted cardiolipin appears to be specifically present only in one genus of the *Halobacteriaceae* family. In particular, S-TGD1-PA, 3-HSO<sub>3</sub>-Galp-β1,6-Manp-α1,2-Glcp-α1,1-[sn-2,3-di-O-phytanyl-glycerol]-6-[phospho-sn-2,3-di-O-phytanyl-glycerol], is present only in *Halobacterium* and S-DGD-5-PA, 2-HSO<sub>3</sub>-Manp-α1,2-Glcp-α1,1-[sn-2,3-diphytanyl-glycerol]-6-[phospho-sn-2,3-diphytanyl-glycerol], only in *Halorubrum*, while the third, 6'-HSO<sub>3</sub>-

D-Manp-α1-2-D-Glcp-α1,1-[sn-2,3-di-O-phytanyl-glycerol]-6-[phospho-sn-2,3-di-O-phytanyl-glycerol] (named S-GL-2) is specific to *Haloferax* (Sprott et al., 2003).

Here we examine the possibility of using NAO as a specific labeling agent for archaeal BPG and possibly also for the substituted cardiolipins of extremely halophilic archaeons by means of fluorescence microscopy and absorption spectroscopy studies. As most ether lipids are not commercially available, the authentic standards here used were isolated and purified in our laboratory. The membranes of extremely halophilic Archaea represent an interesting model to study the binding of NAO to anionic phospholipids; in particular the presence of PGP-Me, carrying two phosphate groups on the polar head (like cardiolipin), but having only two lipid chains (see structure in Fig. 2), can be useful to test whether the bridge structure is a structural determinant for the dye binding.

Although most reported data refers to ether lipids, the present study allows a better understanding of the molecular determinants for the interaction of NAO with phospholipids in general and of its ability to specifically stain membrane domains in Archaea, as well as in bacteria and mitochondria.

## 2. Experimental procedures

### 2.1. Materials

10-N-nonyl acridine orange (NAO) and cardiolipin sodium salt from bovine heart were purchased from Sigma. As NAO is sensitive to light, stock solution (in ethanol) was prepared just prior to use. All organic solvents used were commercially distilled and of the highest available purity (Sigma–Aldrich). Plates for thin-layer chromatography (TLC) (Silica gel 60A, 20 cm × 10 cm, layer thickness 0.2 mm) were obtained from Merck.

### 2.2. Microorganism culture

The microorganisms used throughout this study were the following: (a) the NRC-1 strain *Halobacterium salinarum* (kindly provided by F. Rodriguez-Valera); (b) an isolate closely related to

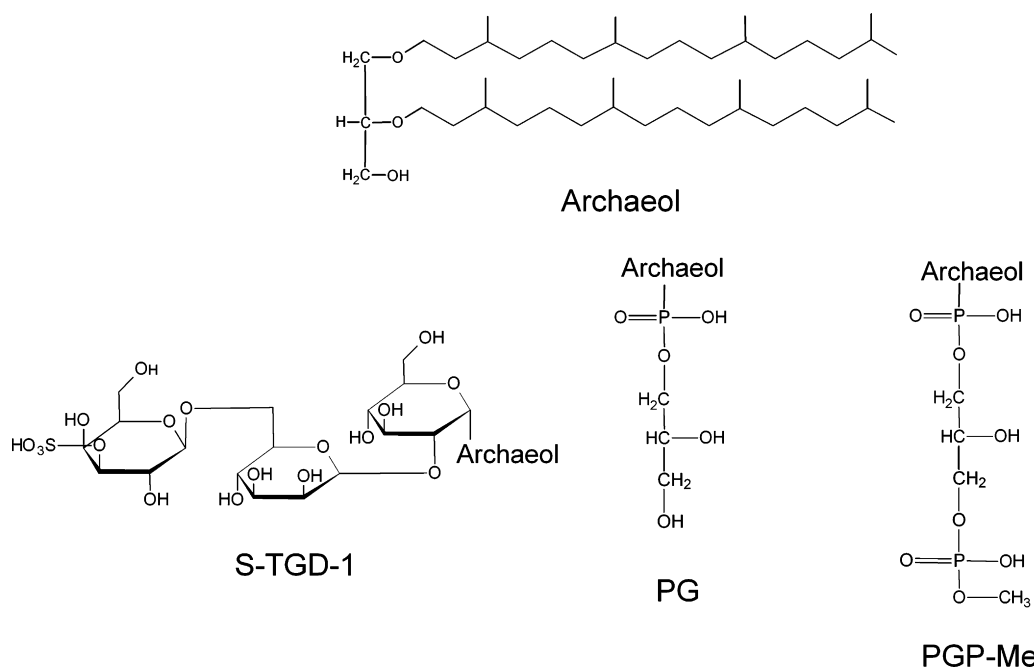
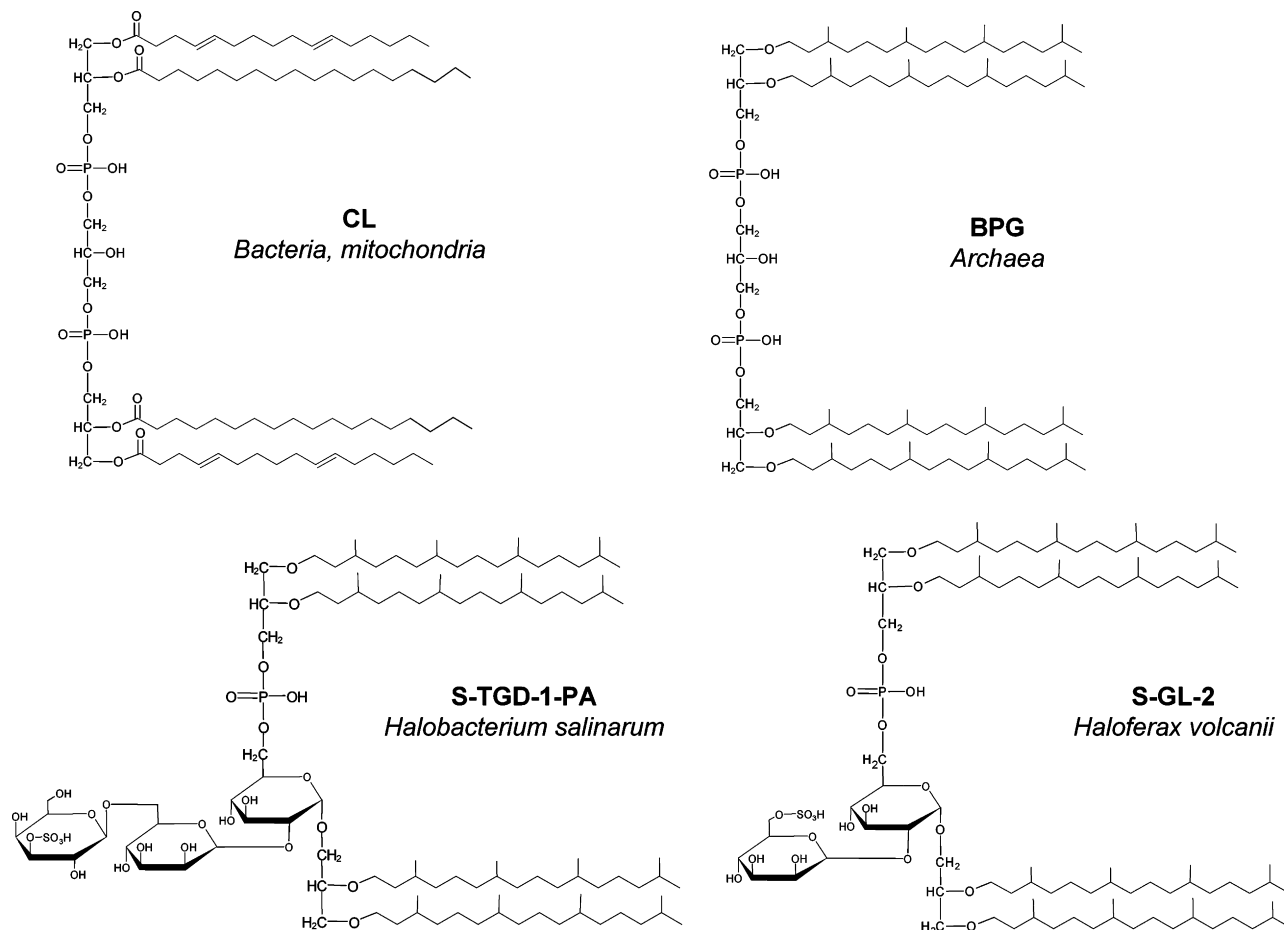


Fig. 2. Structures of some ether lipids. S-TGD-1 is sulfated triglycosylarchaeol (3-HSO<sub>3</sub>-Galp-β1,6-Manp-α1,2-Glcp-α1,1-sn-2,3-diphytanyl-glycerol); PGP-Me, phosphatidyl-glycerophosphate methyl ester; PG, phosphatidylglycerol.



**Fig. 3.** Structures of cardiolipins. CL is the cardiolipin present in bacteria and mitochondria, archaeal BPG (or ether lipid cardiolipin analogue) is found in many extremely halophilic archaeons (Corcelli et al., 2000; Lattanzio et al., 2002); S-TGD-1-PA is present in the purple membranes of *Halobacterium salinarum* (Corcelli et al., 2000), while S-GL-2 is in *Haloferax volcanii* (Sprott et al., 2003).

*Halorubrum trapanicum* sp. (MdS1 strain) isolated from the salterns of Margherita di Savoia, in the South of Italy (Lopalco et al., 2004); (c) the square halophilic archaeon *Haloquadratum walsbyi* HBSQ001 strain (kindly provided by F. Rodriguez Valera).

*Halobacterium* and *Halorubrum* were grown as previously described (Oesterhelt and Stoekenius, 1974; Lopalco et al., 2004), while the square cells were grown in light in a shaker at 80 rpm at 40 °C in HAS medium containing yeast extract (L21, Oxoid), prepared as described in (Bolhuis et al., 2004). Cells at stationary growth phase were harvested by centrifugation and immediately used for lipid extraction.

### 2.3. Membrane isolation

Purple (PM) and red membranes (RM) were isolated from *Halobacterium salinarum* and *Halorubrum trapanicum*, respectively, as previously described (Oesterhelt and Stoekenius, 1974; Lopalco et al., 2004).

### 2.4. Lipid extraction and analysis

Total lipids were extracted from cells and membranes using the Bligh and Dyer method, as modified for extreme halophiles (Kates, 1986). The extracts were carefully dried under N<sub>2</sub> before weighing and then dissolved in chloroform.

Plates for thin-layer chromatography (TLC) were washed twice with chloroform/methanol (1:1, v/v) and activated at 120 °C before

use. Total lipid extracts were analyzed by TLC on silica gel 60A plates in solvent A (chloroform/methanol/90% acetic acid, 65:4:35, by vol.). All lipids were detected by spraying with 5% sulfuric acid, followed by charring at 120 °C (Kates, 1986).

### 2.5. Isolation and purification of individual ether lipids

As most archaeal membrane lipids are not commercially available, we firstly isolated and purified the lipids used in the present study from microorganism cultures (Corcelli and Lobasso, 2006). The isolation of individual lipid components of the total extracts was performed as previously described (Corcelli et al., 2000; Corcelli and Lobasso, 2006).

### 2.6. NAO binding to ether lipid vesicles

Aliquots of chloroform solutions of different lipids (1 mg/mL) were evaporated to dryness in glass tubes under a gentle nitrogen stream. The resulting solvent-free lipid films were then hydrated in a buffer containing 155 mM KCl, 10 mM MOPS, pH 7.2 (KM-buffer), dispersed by hand shaking and incubating in rotary shaker overnight. The vesicles (final lipid concentration 1.5 μM) were incubated in the presence of 3 μM NAO for 5 min at room temperature and absorption spectrum variations at 495 nm/474 nm were measured with an Agilent 8453 diode-array spectrophotometer.

For measurements of NAO fluorescence spectra, 3 μM NAO was added to lipid vesicles, composed of non-archaeal phosphatidyl-

choline (PC) mixed with (a) mitochondrial CL, (b) archaeal BPG or (c) the archaeal glycolipid S-DGD-5 (final concentration PC 6  $\mu\text{M}$ , other lipids 3  $\mu\text{M}$ ). After 5 min incubation at room temperature the fluorescence emission was measured at 640 nm with an excitation wavelength of 450 nm (emission and excitation band widths were 5 nm) with a Jasco FP-6200 spectrofluorometer. The emission spectrum of unbound dye in the supernatant was measured after centrifugation at  $150,000 \times g$  for 60 min.

### 2.7. NAO binding to isolated purple and red membranes

Increasing amounts of membranes (PM or RM) were added to 3 mL of 3  $\mu\text{M}$  NAO in a buffer containing 50 mM  $\text{K}_2\text{HPO}_4$ , 50 mM  $\text{KH}_2\text{PO}_4$ , pH 7.2 (P-buffer). After mixing vigorously and incubating for 5 min, absorption spectra variations at 495 nm/474 nm of the samples were measured spectrometrically.

### 2.8. Preparation of giant phospholipid vesicles (GPVs)

GPVs were prepared at room temperature (23 °C) by the electroformation method (Angelova et al., 1992) with small modifications as described in (Tomsie et al., 2005). Appropriate volumes of chosen lipids, all dissolved in a 2:1 chloroform/methanol mixture, were combined in a glass jar and thoroughly mixed. 10  $\mu\text{L}$  of lipid mixture was applied to platinum electrodes. The solvent was allowed to evaporate in a low vacuum for 2 h. The coated electrodes were placed in the electroformation chamber, which was then filled with 3 mL of 0.2 M sucrose solution. An AC electric current with an amplitude of 5 V and a frequency of 10 Hz was applied to the electrodes for 2 h, which was followed by 2.5 V and 5 Hz for 15 min, 2.5 V and 2.5 Hz for 15 min and finally 1 V and 1 Hz for 15 min. The content was rinsed out of the electroformation chamber with 4 mL of 0.2 M glucose and stored in a plastic test tube. The vesicles were left for sedimentation under gravity for one day at 4 °C. 50  $\mu\text{L}$  of the sediment was collected from the bottom of the tube and put into the observation chamber.

### 2.9. Fluorescence microscopy

For cell staining experiments, NAO was added to a final concentration of 100 nM to a *Haloquadratum walsbyi* culture in growth medium at the stationary growth phase. After 1 h of incubation at room temperature, the cells were viewed in the presence of the dye with a Leica epifluorescence microscope equipped with a 100 $\times$  fluorite oil immersion objective and a standard fluorescein isothiocyanate (FITC) filter set. Images were captured with a cooled charge-coupled camera device and manipulated in Iplab and Adobe Photoshop 7.0.

In control experiments, NAO nanomolar concentrations (nM 100–200) were added to *Haloquadratum walsbyi* cells in order to check whether these concentrations could interfere with the growth and functionality of the cells.

For GPV staining experiments, 1  $\mu\text{L}$  of 0.5  $\mu\text{M}$  NAO in ethanol solution was left to evaporate and then 50  $\mu\text{L}$  of vesicles, in sugar solution, were added. We then waited for 2–3 min until the NAO dye was taken up by the lipids. Then the whole sample was put on the cover glass, which was covered by another cover glass and sealed with grease. Observation with a Zeiss Axiovert 200 inverted microscope was performed 3–15 min after the addition of NAO to the vesicles in sugar solution. The excitation wavelength was 500 nm, while the emission wavelength was 535 nm. Exposure time was 1000 ms.

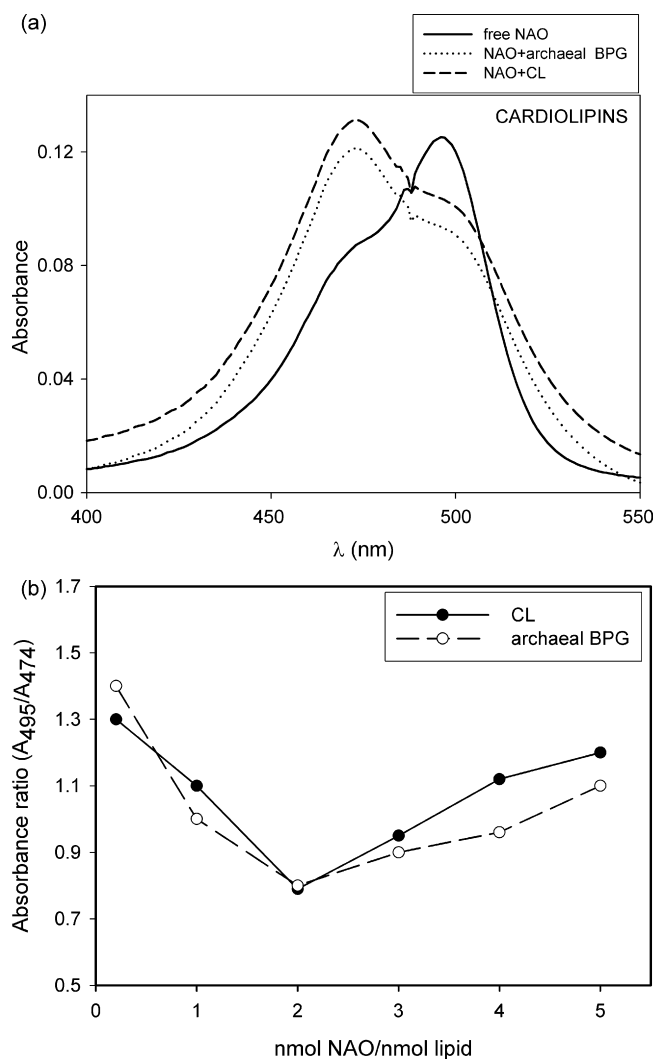
## 3. Results

### 3.1. Binding of NAO to ether lipid vesicles

It has been reported that when NAO binds to cardiolipin, absorption spectral changes and fluorescence emission occur as a consequence of dye dimer formation (Petit et al., 1992; Gallet et al., 1995).

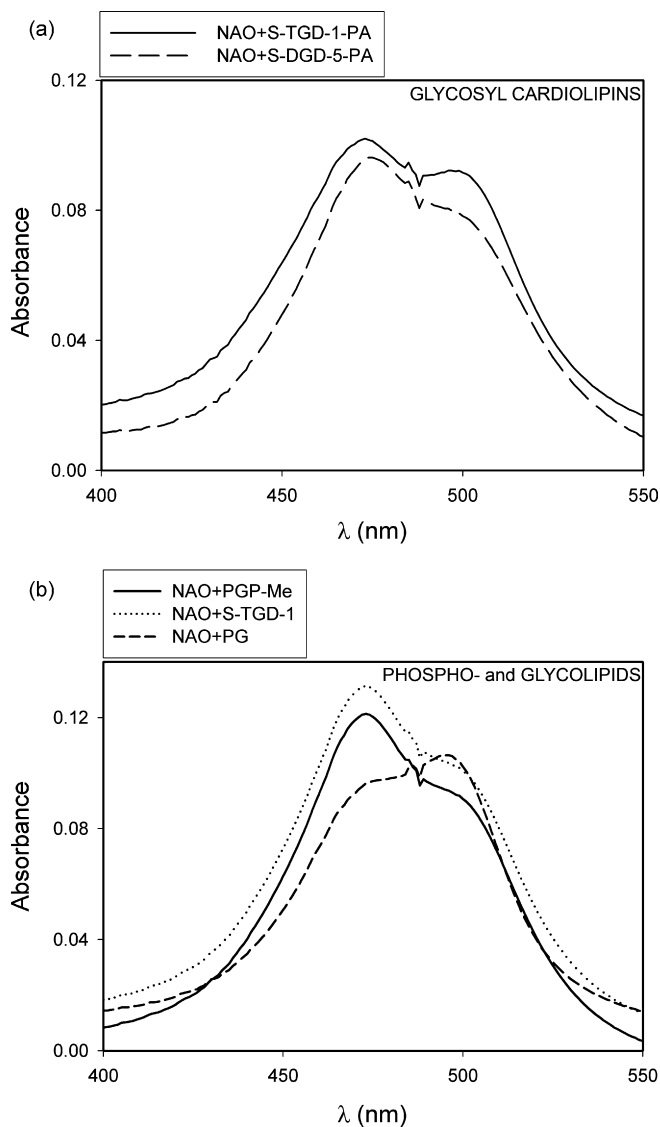
At low concentration ( $\mu\text{M}$ ) the absorption spectrum of free monomeric NAO is maximal at 495 nm and presents a shoulder at 474 nm, while in the presence of cardiolipin (or at higher dye concentrations) NAO absorption at 495 nm decreases and the maximum is shifted to 474 nm: the decrease in absorption corresponds to the disappearance of dye monomers, while the increase at 474 nm indicates the formation of NAO dimers. It has also been previously shown that when other phospholipids are added to free NAO, no significant spectral changes can be seen (Petit et al., 1992).

Fig. 4a shows the absorption spectrum of 3  $\mu\text{M}$  free NAO and the spectra of NAO bound to either cardiolipin (CL) or ether lipid cardi-



**Fig. 4.** Comparison of NAO interaction with either cardiolipin (CL) or the ether lipid cardiolipin analogue (archaeal BPG). (a) Absorption spectra of: (full line) 3  $\mu\text{M}$  free NAO; (dotted line) a mixture of NAO+CL vesicles; (dashed line) a mixture of NAO+archaealBPG vesicles. In the experiments cardiolipins were at a concentration of 1.5  $\mu\text{M}$  and NAO was 3  $\mu\text{M}$ , both in KM-buffer. (b) Evolution of the absorption ratio (495 nm/474 nm). Increasing either CL or BPG concentrations were added to 3  $\mu\text{M}$  NAO and then absorption measurements were performed at 495 and 474 nm.

olipin analogue, two lipid molecules having a similar backbone, the same head group but different lipid tails (see structures in Fig. 3). Since it has been reported that the stoichiometry of the binding NAO/cardioliolipin is 2 mol NAO/1 mol CL (Petit et al., 1992), we used appropriate amounts of lipids to operate in the same molar ratio. It can be seen that ether lipid cardioliolipin (archaeal BPG) is able to bind NAO in the same manner as CL. Monitoring the evolution of the 495 nm/474 nm absorbance ratio, when increasing amounts of lipids were added to a fixed NAO concentration, showed that a minimum value was obtained when two dye molecules were bound to one CL molecule (Fig. 4b) and the same behavior was shown by ether lipid archaeal BPG. These results are identical to those previously obtained by Petit et al. (1992). Furthermore, the changes in the NAO spectrum following the addition of two ether lipid glycosyl-cardioliolipin analogues are shown in Fig. 5a. It can be seen that both the ether lipid cardioliolipin analogues S-TGD-1-PA and S-DGD-5-PA were also able to give rise to the shift of the NAO peaks at NAO/lipid ratio of 2. Assuming that NAO peak shift in the absorption spectrum corresponds to the dimer formation, this result could be simply



**Fig. 5.** NAO interaction with ether glycosyl cardioliolipins (a) and phospho- and glycolipids (b). Absorption spectra of: (a) a mixture of NAO+S-TGD-1-PA and NAO+S-DGD-5-PA vesicles and (b) a mixture of NAO+PGP-Me, NAO+PG, and NAO+S-TGD-1 vesicles. The experimental conditions were the same as in Fig. 4a.

explained by suggesting that NAO can form dimers not only in the presence of two phosphate groups, but also in the presence of one phosphate together with a sulfate residue in the polar head of the lipid molecule. The position in space of the two non-identical acid residues in the asymmetric head group of these complex molecules could be similar to that of the two phosphate residues in archaeal BPG, possibly also due to constraints determined by the presence of the bridge structure.

To test this possibility we checked whether PGP-Me, PG and the sulfoglycolipids could also induce NAO shift; in previous studies we showed that these lipids are the precursors or the monomeric counterpart of archaeal BPG and glycosyl-cardioliolipins in the Archaea (Lopalco et al., 2004; Lobasso et al., 2003).

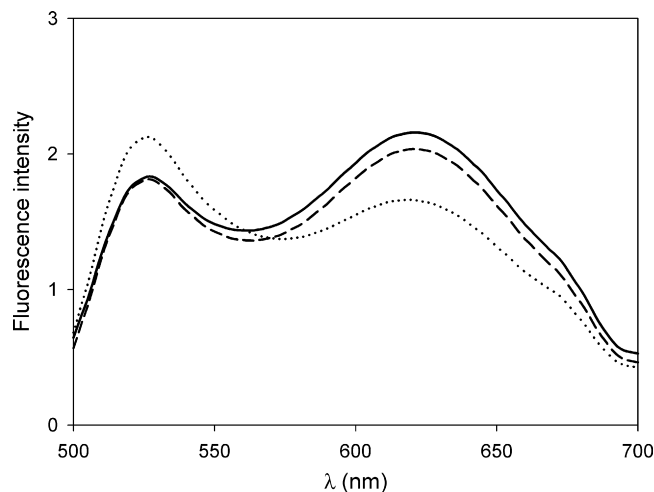
We found that PGP-Me induces NAO shift in the absorbance spectrum, as well as PGS (not shown), but not PG (Fig. 5b). These results suggest that the presence of two acidic groups is required to determine NAO absorbance shift, while the number of hydrophobic chains or the presence of the bridge structure is not important.

In addition, we surprisingly found that sulfoglycolipids are also able to induce the peak shift in the absorbance spectrum (Fig. 5b). As these monomeric sulfolipids have one acidic residue in the polar head, this finding can be explained by considering that specific hydrophobic stacking interactions between the aromatic ring of NAO and carbohydrates of the polar head of the sulfoglycolipid could contribute to dimer formation.

Table 1 summarizes the above results regarding the interactions of NAO with various ether phospholipids and glycolipids, together with their structural features.

In parallel experiments we measured NAO fluorescence in the presence of archaeobacterial lipids. When NAO interacts with cardioliolipin, the dye excitation and emission wavelengths shift from 496 and 525 nm to 450 and 640 nm, respectively (Petit et al., 1994; Gallet et al., 1995).

Fig. 6 shows the NAO red fluorescence emission measured in representative experiments, in which NAO was added to non-archaeal phosphatidylcholine (PC) vesicles containing (a) mitochondrial CL, (b) archaeal BPG, or (c) the archaeal glycolipid S-DGD-5. Furthermore, after spectra recording, the different samples were centrifuged to check for unbound NAO. Since the lipids in those experiments were saturating, no free NAO was left in the super-



**Fig. 6.** NAO fluorescence spectra in the presence of lipids. 3  $\mu$ M NAO was incubated with lipid vesicles containing non-archaeal phosphatidylcholine (PC) plus either mitochondrial cardioliolipin (CL) (dashed line), or archaeal BPG (dotted line), or archaeal glycolipid S-DGD-5 (full line). The vesicle PC/other lipid ratio was 2:1, by weight, in all samples; CL, BPG and S-DGD-5 were 3  $\mu$ M. The fluorescence emission was measured at 640 nm with an excitation wavelength of 450 nm.

**Table 1**  
The structural features of the studied archaeal lipids and their interactions with NAO.

Lipid	NAO binding	Number of phytanyl chains	Number of residues on the polar heads		
			Sugars	Phosphates	Sulfates
BPG	+	4	0	2	0
S-TGD-1-PA	+	4	3	1	1
S-DGD-5-PA	+	4	2	1	1
S-TGD-1	+	2	3	0	1
S-DGD-5	+	2	2	0	1
S-DGD-1	+	2	2	0	1
PGP-Me	+	2	0	2	0
PGS	+	2	0	1	1
PG	–	2	0	1	0

natants (not shown). The spectrum of NAO/CL containing vesicles shows the peak at 640 nm diagnostic for the presence of NAO/CL specific interaction together with a small peak of green fluorescence emission. It can be seen that, as well as CL, either archaeal BPG or S-DGD-5 can induce the red shift of fluorescence emission.

The experiments shown in this section suggest that neither the blue shift in the absorbance spectrum nor the red shift in the fluorescence emission can be used as qualitative criteria to distinguish between the NAO interactions with archaeal BPG and those with other archaeal lipids.

### 3.2. Binding of NAO to membranes isolated from extremely halophilic archaeons

We selected two well-known membrane preparations from archaeal halophilic microorganisms: the purple membranes of *Halobacterium salinarum* and the red membranes of *Halorubrum trapanicum* (Corcelli et al., 2000, 2002, 2007; Lopalco et al., 2004).

Fig. 7a illustrates the TLC analysis of the lipid extracts of the two membrane preparations. They contain the same phospholipids PGS, PGP-Me and PG, but different kinds of cardiolipins and glycolipids. The so-called glycardiolipin S-TGD1-PA is a major lipid component of the purple membrane, while archaeal BPG is highly enriched in the red membrane.

When appropriate amounts of either PM or RM were added to free NAO, absorption spectra showed NAO blue shift (Fig. 7b). By following the evolution of the absorption ratio at increasing amounts of PM or RM, we found that the ratio reached the same minimum value at the NAO/total lipids molar ratio of approximately 2 (Fig. 7c). These results indicate that, despite the difference in lipid composition, NAO establishes similar interactions with PM and RM, and, in agreement with previous observations, suggest that the dye may interact as dimers not only with cardiolipin, but also with other acidic ether lipids.

### 3.3. NAO fluorescence staining of *Haloquadratum walsbyi* cells and giant liposomes

To study the NAO binding to archaeal membranes *in vivo*, we selected the extremely halophilic square-shaped microorganism *Haloquadratum walsbyi*, which has the following characteristics: (a) absence of both cardiolipin and cardiolipin analogues in the membrane and (b) large planar cell surface allowing easy visualization of membrane staining.

Fig. 8a shows phase-contrast microscopic images of square cells rich in intracellular refractive bodies (gas vesicles). Panel b in the same figure illustrates the ESI-MS and TLC lipid profiles of these cells. The ESI-MS diagnostic peaks for ether lipid cardiolipins and glycosyl-cardiolipin analogues are absent, while PGP-Me and S-DGD-5 appear to be abundant lipid components in the microorganism membranes.

The picture of *Haloquadratum walsbyi* after incubation with NAO is shown in Fig. 9a. It can be seen that the distribution of the dye is not homogeneous; a fine patch of green fluorescent domains can be clearly observed over the large planar surface of the cell.

In order to exclude any interference of the highly refractive gas vacuoles in the fluorescent image, square cells were also centrifuged before NAO staining. In fact it is well known that the gas vesicles collapse after centrifugation, while the cell shape remains unmodified (Walsby, 1980). NAO fluorescence pattern of square cells after vacuole collapsing was found to be similar to that in Fig. 9a.

As *Haloquadratum walsbyi* membranes do not contain cardiolipins (see Fig. 8b), we conclude that the areas of NAO fluorescence in these cells do not represent the cardiolipin membrane domains and that in general NAO staining in extremely halophilic archaeal cells is not specific for cardiolipin.

The uneven distribution of the NAO green fluorescence clearly shows that there are areas of the archaeobacterial membranes with a different affinity for the dye. This might depend on the quality of the lipids present in the various membrane domains as well as on the density of some particular lipids and/or the lipid/protein ratio. Dark or non-fluorescent areas could represent the PG domains, where the density of the blue shift-inducing lipids would be lower than in the fluorescent domains.

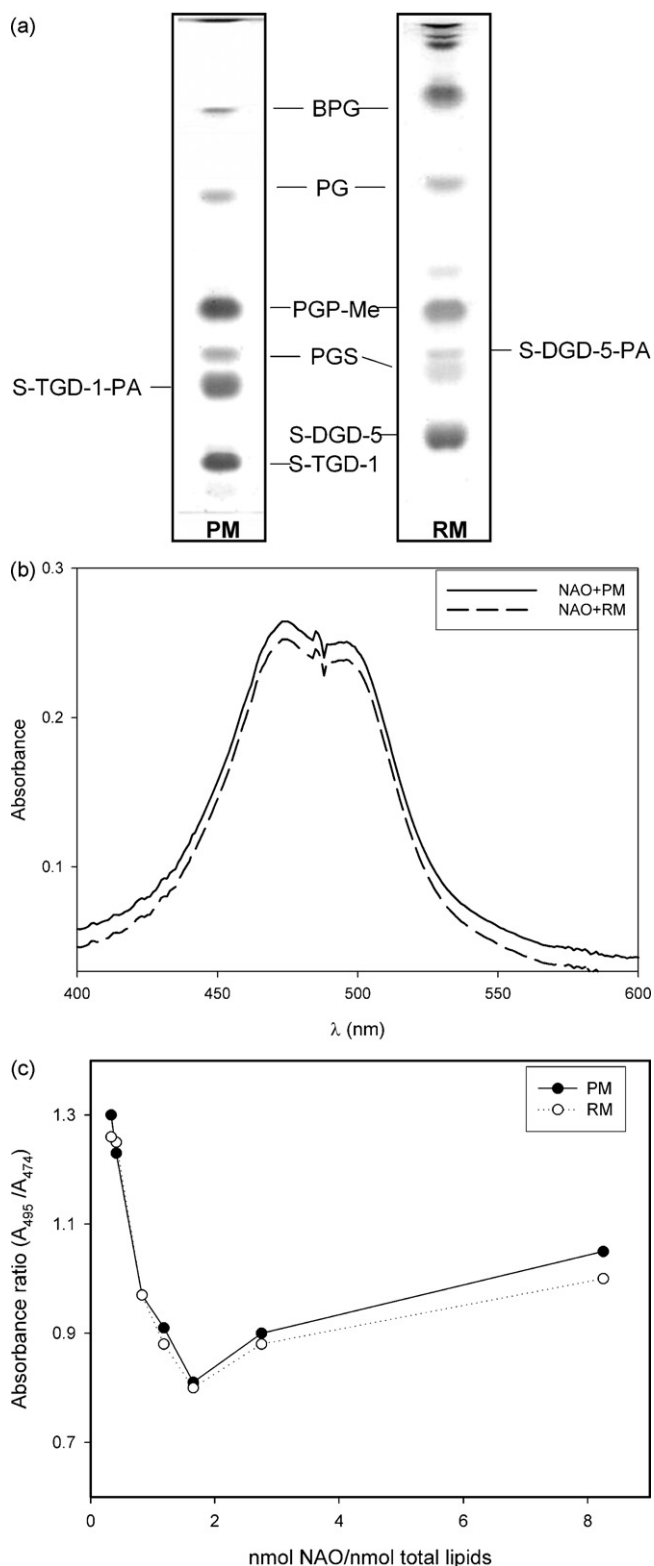
To better interpret the NAO green fluorescence of the *Haloquadratum walsbyi*, we examined NAO staining of artificial membrane giant vesicles (giant liposomes) constituted of archaeobacterial lipids. The use of giant liposomes is particularly convenient, because it allows the study and detection of the NAO binding by fluorescence microscopy.

Fig. 9b shows the green fluorescence of giant unilamellar vesicles containing archaeal BPG; in particular, the left panel illustrates the non-uniform distribution of green fluorescence in giant liposomes consisting of non-archaeal phosphatidylcholine (PC) and cholesterol plus ether lipid archaeal BPG (3:1:1, by weight). Similar results were obtained by substituting archaeal BPG with mitochondrial cardiolipin; while in the absence of archaeal BPG, the PC/cholesterol giant vesicles were not stained by the fluorescent dye (not shown).

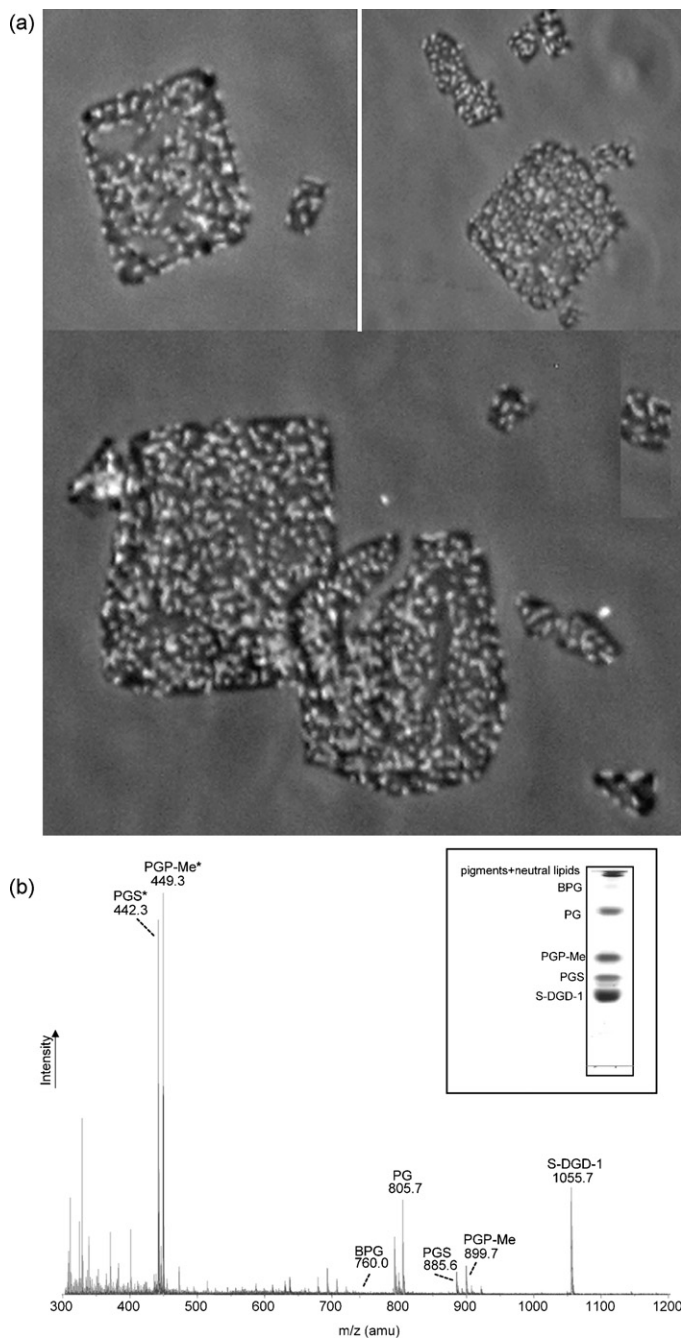
The right panel of the same figure illustrates the fluorescence of giant liposomes consisting of archaeal polar lipids alone, S-DGD-5:BPG (4:1, by weight). In this picture the fluorescence is homogeneously distributed; giant liposomes made of S-DGD-5 alone are also fluorescent under the same experimental conditions (not shown).

Data in Fig. 9 indicate that NAO not only fluoresces with archaeal BPG, but also with other ether lipids, again suggesting that it is difficult to use NAO as a specific labeling agent in archaeobacterial membranes.

In recent literature reports dealing with the localization of bacterial cardiolipin, it has been demonstrated that NAO green flu-

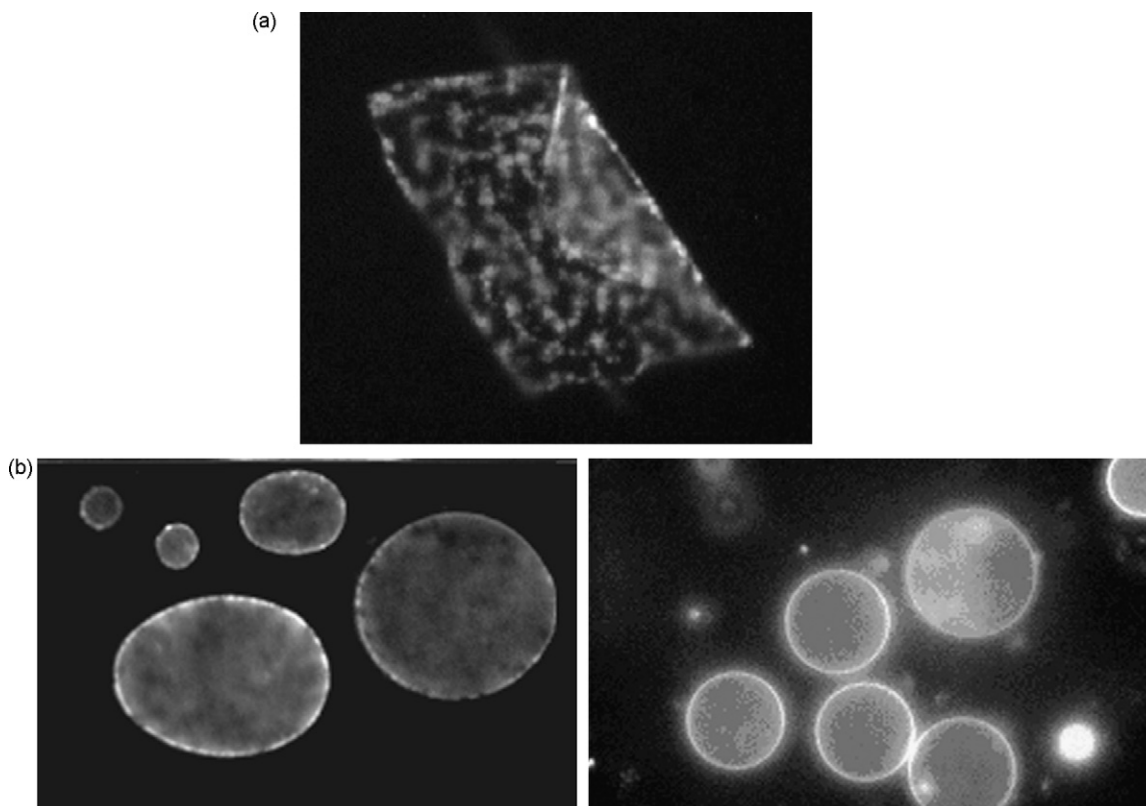


**Fig. 7.** NAO interaction with archaeal isolated membranes. (a) Comparison of the lipid profiles of purple membrane and red membrane. Total lipids were extracted from purple membrane isolated from *Halobacterium salinarum* (NRC-1 strain) and red membrane isolated from *Halorubrum* sp. (Mds1 strain), as described in Section 2. 70  $\mu$ g of lipid extract were loaded on each lane. The abbreviated names of individual lipid components in the extracts are reported. Phospholipids: PG, PGP-Me and PGS in both the membranes; glycolipids: S-TGD-1 in PM and S-DGD-5 in RM; cardiolipin analogues: S-TGD-1-PA in PM, S-DGD-1-PA in RM and archaeal BPG in both PM and RM. (b) Absorption spectra of a mixture of NAO + PM (dotted line) and NAO + RM



**Fig. 8.** Microscopic observation (a) and lipid analysis (b) of *Haloquadratum walsbyi* (HBSQ001 strain) cells. (a) Phase-contrast microscopic images of square cells rich in gas vesicles. The cells in fresh culture are about 40  $\mu$ m  $\times$  40  $\mu$ m wide, but extremely thin (only 0.1–0.5  $\mu$ m thick). Magnification was 1000 $\times$ . (b) Cells were harvested by centrifugation and total lipids were extracted (as described in Section 2) and analyzed by electro-spray ionization-mass spectrometry (ESI-MS) and thin-layer chromatography (TLC) (inset). In the negative-ion ESI-MS spectrum of total lipid extract of cells, the \* indicates bicharged peaks; 80  $\mu$ g of lipid extract were loaded in the TLC. The abbreviated names of the individual lipid components present in the extract have been reported.

(dashed line). In these spectra the NAO/total lipid molar ratio was approximately 2. (c) Evolution of the absorption ratio (495 nm/474 nm). Increasing amounts of either PM or RM were added to 3  $\mu$ M NAO.



**Fig. 9.** NAO staining of living square cells (a) and giant unilamellar vesicles (b) with NAO. (a) NAO was directly added to the cells in the growth medium and the picture (magnification 1000 $\times$ ) was taken after 1 h of incubation with the dye (100 nM) by using a standard FITC filter set. (b) 10 nM NAO was added to 50  $\mu$ L of giant unilamellar vesicles in sugar solution. Observation was performed 3–5 min after the addition of NAO to the vesicles in sugar solution. The vesicles were consisted of: (left panel) phosphatidylcholine:cholesterol:BPG (3:1:1, by weight) and (right panel) S-DGD-5:BPG (4:1, by weight). Excitation wavelength was 500 nm, while emission wavelength was 535 nm. Exposure time was 1000 ms.

orescence represents the areas at low cardiolipin density, while the NAO red fluorescence indicates the domains at high cardiolipin content where NAO multimers can be formed (Mileykovskaya, 2007; Romantsov et al., 2007).

Preliminary measurements of the NAO fluorescence in giant liposomes consisting of archaeobacterial lipids by confocal microscopy showed that, at low NAO concentrations, the red fluorescence emission is comparable or higher than the green fluorescence (Michelangelo Corcelli, unpublished results).

#### 4. Discussion

The ether lipids of archaeal microorganisms present many distinct features compared to the most commonly known diacylglycerol derivatives present in bacterial and eukaryotic membranes. Notwithstanding their distinctive chemical elements, the general molecular architecture of archaeal membrane ether lipids is quite similar to that of the most commonly known phospholipids. As an example the glycosyl cardiolipins present in the Archaea have the same backbone as their analogues in bacteria (Gutberlet et al., 2000).

We have undertaken the study of the binding of NAO to ether lipids of extremely halophilic archaeal microorganisms to check whether this fluorescent dye can be useful to specifically label cardiolipin-enriched membrane domains and, at the same time, to gain further insight into the general mechanism of NAO interaction with membrane phospholipids.

The high affinity binding of the dye for cardiolipin in bacterial and mitochondrial membranes has been explained by two essential interactions: (1) the electrostatic interaction between the

quaternary ammonium of nonyl acridine orange and the ionized phosphate residues of cardiolipin and (2) hydrophobic interactions between adjacent chromophores. It has been proposed that these two interactions result in the formation of NAO dimers on the polar head of cardiolipin and no other phospholipids (Petit et al., 1992).

In a refined model it has been suggested that the nonyl group inserts itself in the bilayer at the hydrophobic surface generated by the presence of four chains in the phospholipid. Because CL contains four chains and its head group appears to be compact, the NAO ring system could fit between the CL molecules in aggregate stacks (Mileykovskaya et al., 2001).

Here we show that NAO dye exhibits specific blue shift in absorbance and red shift in fluorescence when bound to archaeobacterial anionic phospholipids and glycolipids. Taken all together, absorption spectroscopy data obtained in artificial self-organized lipid vesicles (summarized in Table 1) indicate that NAO establishes similar interactions with a number of archaeobacterial phospholipids and glycolipids including the archaeal cardiolipin analogue BPG. In other words, different from bacteria and mitochondria, in archaeal microorganisms NAO does not preferentially bind to BPG.

The molecular determinants to induce the blue absorption shift include the presence on the polar head of (a) two acidic groups or (b) one acidic group plus carbohydrate residues, whereas neither the bridge structure nor the number of hydrophobic chains are important.

In addition, absorption spectroscopy findings indicate that the NAO interaction with archaeal phospholipids is similar to that occurring with mitochondrial cardiolipin. This evidence is somewhat surprising, as it has been reported in the literature that the mode of NAO binding with mitochondrial cardiolipin is unique, due



to the peculiar conformation of its head group, which is highly compact due to the presence of H-bonds between two phosphates.

In cardiolipin-rich domains the tight array of cardiolipin molecules in the bilayer allows a perfect fit of cardiolipin multimers over the lipid surface (Mileykovskaya et al., 2001); whereas in the case of archaeal BPG the head group packing is expected to be less compact because of phytanyl chains.

We have also studied the NAO binding to membranes isolated from extremely halophilic microorganisms, representing a naturally organized lipid bilayer, *in vitro*. Data suggest that NAO stacks uniformly over the membrane surface of both the purple membrane and the red archaeobacterial membrane, and that NAO binding cannot be used to show either the presence of cardiolipins or fine differences between the lipid compositions of these two membrane domains.

The first conclusion was confirmed by the result of NAO staining a square cell, representative of archaeal extremely halophilic microorganisms, having very small amounts of cardiolipin. Clearly, the fine fluorescence pattern present all over the large planar surface of *Haloquadratum walsbyi* cannot represent the distribution of cardiolipin membrane domains. As an alternative, the NAO fluorescence pattern in the square cells might be considered a useful map of the membrane regions with higher negative charge density.

Finally, results obtained by studying the NAO binding with giant liposomes consisting of archaeobacterial phospholipids confirmed that NAO fluorescence might be the consequence not only of the presence of archaeal BPG but also of other archaeobacterial polar lipids. Experiments are in progress to investigate the presence and distribution of red NAO fluorescence in square cells and giant liposomes consisting of archaeobacterial lipids, in order to fully elucidate the nature and mechanisms of NAO interaction with archaeobacterial phospholipids as well as the physiological significance of the NAO fluorescence map in *Haloquadratum walsbyi* cells.

Although the dye cannot be utilized to label BPG domain in archaeal cells due to the interference of other anionic phospho- and glycolipids, it might still be potentially useful to label microdomains in artificial membranes consisting of lipid matrix with a low affinity for the dye.

## Acknowledgements

We thank R. Roberto and M. Rocchi (Dipartimento di Genetica e Microbiologia, Università degli Studi, Bari, Italy) for the cell fluorescence microscopy, and G. Mascolo (CNR-IRSA, Bari, Italy) for ESI-MS analysis. The work was supported by the Ministero Italiano dell'Università e della Ricerca (Fondi d'Ateneo), the Ministero Italiano della Difesa (contract no. 1999/13.12.2005) and the Istituto per i processi chimico-fisici del Consiglio Nazionale delle Ricerche, Bari, Italy (CNR-IPCF).

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