Lipid-protein stoichiometries in a crystalline biological membrane: NMR quantitative analysis of the lipid extract of the purple membrane

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Abstract The lipid/protein stoichiometries of a naturally crystalline biological membrane, the purple membrane (PM) of *Halobacterium salinarum*, have been obtained by a combination of ^{31}P- and ^{1}H-NMR analyses of the lipid extract. In total, 10 lipid molecules per retinal were found to be present in the PM lipid extract: 2–3 molecules of phosphatidylglycerophosphate methyl ester (PGP-Me), 3 of glycolipid sulfate, 1 of phosphatidylglycerol, 1 of archaeal glycocardiolipin (GlyC), 2 of squalene plus minor amounts of phosphatidylglycerosulfate (PGS) and bisphosphatidylglycerol (archaeal cardiolipin) (BPG) and a negligible amount of vitamin MK8. The novel data of the present study are necessary to identify the lipids in the electron density map, and to shed light on the structural relationships of the lipid and protein components of the PM.—Corcelli, A., V. M. T. Lattanzio, G. Mascolo, P. Papadia, and F. Fanizzi. **Lipid-protein stoichiometries in a crystalline biological membrane: NMR quantitative analysis of the lipid extract of the purple membrane. *J. Lipid Res.* 2002.** 43: **132–140.**

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In the literature there are numerous reports indicating the importance of particular lipids on the activity of a wide range of integral membrane proteins, but so far only few crystallographic studies describing the structural details of protein-lipid interface in membrane protein crystals are available. In the unique case of bacteriorhodopsin (BR), molecular details of the protein-lipid interface can be obtained not only from diffraction patterns of crystals (1–3), but also from direct crystallographic studies of its natural environment, the purple membrane (PM) (4, 5). The PM of *Halobacterium salinarum*, being a 2D crystalline lattice formed only by bacteriorhodopsin and a small number of lipid molecules, is considered one of the best models for studying the mode of assembly of the integral membrane proteins and membrane topography (6).

BR, a seven-transmembrane domain protein, is a light-driven proton pump, which has the retinal as prosthetic group covalently linked to K216 as a protonated Schiff base (7, 8). The main interest of PM studies in the last 3 decades has been the elucidation of the BR structure and in particular of its retinal-containing pocket. So far, the knowledge of the interactions of α-helices with surrounding lipids and the identification of annular lipids is still incomplete, as is the localization of the lipid head groups (1, 2, 5).

In the PM, BR is organized in trimers; to gain a complete knowledge of membrane topography it is necessary to determine the distribution and localization of various lipids in the central cylindrical compartment of trimers and the outer continuous bulk phase. The structures of all polar and neutral lipids presently identified in the PM are reported in **Fig. 1**. It can be seen that PM polar lipids are derivatives of a glycerol diether, 2,3-di-*O*-phytanyl-*sn*-glycerol (archaeol), this basic structure being characteristic of lipids of the cellular membrane of all microrganisms belonging to the kingdom of Archaea (9). The chemical nature of archaeal lipids contributes significantly to the preservation of the structural and functional integrity of the PM under a wide range of temperatures and pH.

Abbreviations: BPG, archaeal cardiolipin or bisphosphatidylglycerol; BR, bacteriorhodopsin; ESI-MS, electro-spray ionization mass spectrometry; GlyC, archaeal glycocardiolipin or 3-HSO_3-Galp-β1,6-Manp-α1,2-Glcp-α1,1-[sn-2,3-di-*O*-phytanylglycerol]-6-[phospho-sn-2,3-di-O-phytanylglycerol]; HPTLC, high-performance thin-layer chromatography; PG, phosphatidylglycerol (diphytanylglycerol ether analog); PGP-Me, phosphatidylglycerophosphate methyl ester (diphytanylglycerol ether analog); PGS, phosphatidylglycerosulfate (diphytanylglycerol ether analog); PM, purple membrane; S-TGD-1 (also named S-TGA-1 by other authors), 3-HSO_3-Galp-β1,6-Manp-α1,2-Glcp-α1,1-sn-2,3-diphytanylglycerol.

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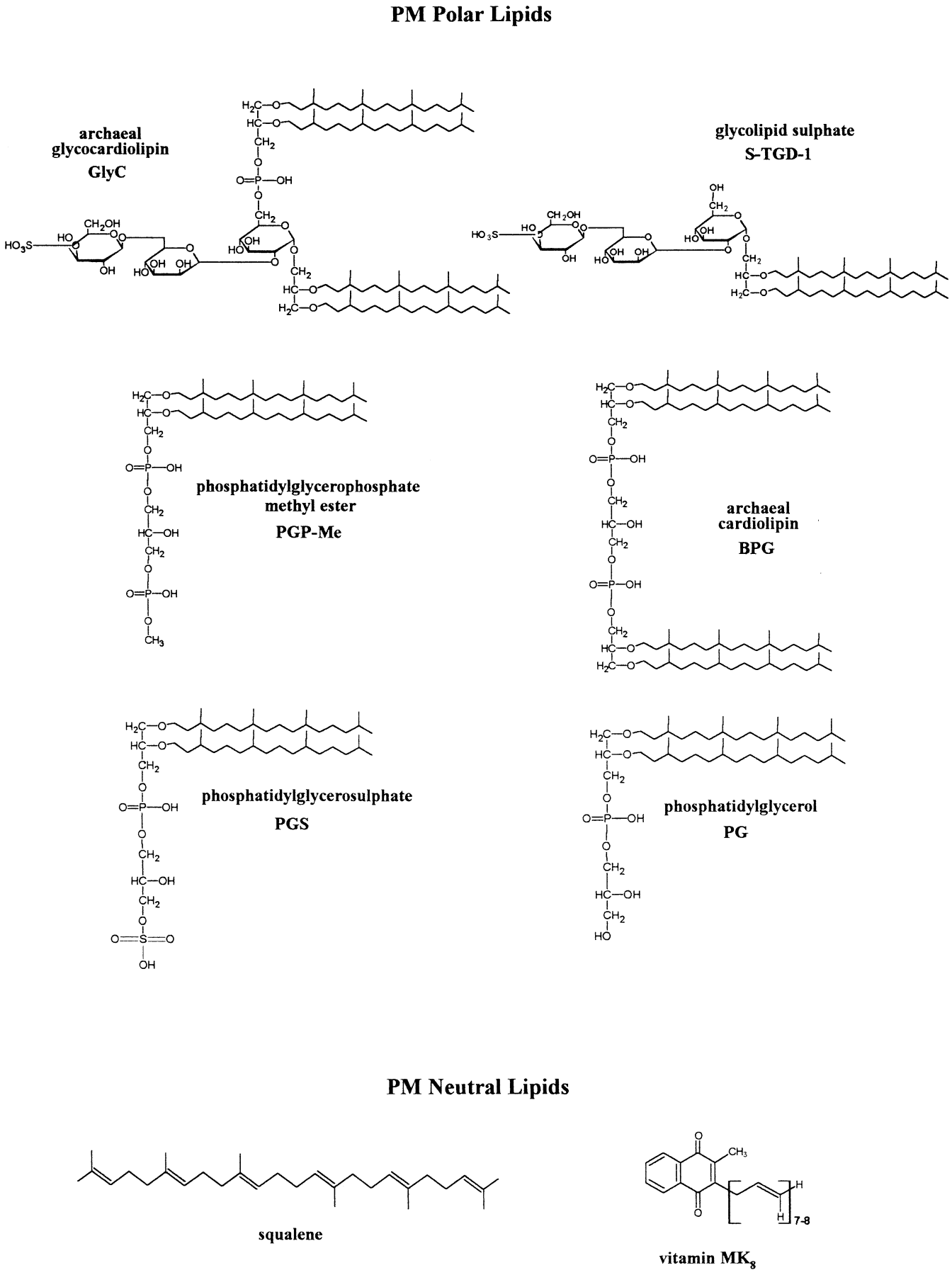

Fig. 1. Structure of the purple membrane (PM) polar and neutral lipids.

Until now, the lipid assignments in structural studies have been based on data of the lipid composition of the purple membrane previously reported by Kates and coworkers (10–12).

Recently, the analysis of residual lipids specifically associated with "delipidated" BR has revealed the presence of a new glycolipid and a new phospholipid in the PM, in addition to the phospholipids, phosphatidylglycerophosphate methyl ester (PGP-Me), phosphatidylglycerol (PG), and phosphatidylglycerosulfate (PGS) and glycolipid (S-TGD-1) previously reported (13). The novel glycolipid and phospholipid were shown, by chemical degradation, mass spectrometry and NMR analyses, to have the structure, respectively, of a phosphosulfoglycolipid, 3-HSO_3-Gal*p*-β1, 6Man*p*-α1,2Glc*p*-α1,1-[*sn*-2,3-di-*O*-phytanylglycerol]-6-[phospho-*sn*-2,3-di-*O*-phytanylglycerol], and of a glycerol diether analog of bisphosphatidylglycerol, *sn*-2,3-di-*O*-phytanyl-1-phosphoglycerol-3-phospho-*sn*-2,3-di-*O*-phytanylglycerol. Interestingly, both novel lipid molecules are analogs of eukaryal cardiolipins; in the following we will refer to them as archaeal glycocardiolipin (GlyC) and archaeal cardiolipin (BPG), respectively. In consideration of the finding of two novel lipids in the PM, it is necessary to reanalyze the overall PM lipid composition, and in particular to reestimate the molar ratio of each individual PM lipid to BR. It has recently been reported that NMR spectroscopy permits the rapid quantitative analysis of lipid extracts from membranes or tissues, representing a valid alternative to the conventional multistep TLC approaches of lipid analysis (14). The application of NMR spectroscopy appears to be particularly suitable to the analysis of the PM lipid extract, because it contains less than 10 different kinds of lipid molecules.

Here we show that, by combining data obtained from ^{31}P-NMR and ^{1}H-NMR spectra of the total lipid extract of PM, it is possible to perform an almost complete qualitative and quantitative analysis of PM lipids, obtaining the relative proportions of different lipids in the extract. In addition, because retinal, the prosthetic group of BR is also present in the lipid extract, our experimental approach allows simple and direct estimation of the lipid/BR stoichiometries in the PM. These data are obviously necessary for the elucidation of the positions of specific lipids in the PM lattice, and possibly also for the identification of endogenous lipids into BR crystals. The method here described can be of general utility in the study of the lipid/protein stoichiometries in highly specialized biological membrane patches.

EXPERIMENTAL PROCEDURES

Materials

DNase was obtained from Sigma. All organic solvents used were commercially distilled and of the highest available purity (Sigma-Aldrich). TLC plates (60A) and high-performance thin-layer chromatography (HPTLC) (60A) plates, obtained from Merck, were washed twice with chloroform-methanol (1:1, v/v) and activated at 120°C before use. Retinal and squalene standards were obtained from Sigma.

Microrganism cultures

An engineered high-producing BR strain (L33) of *Halobacterium salinarum*, the kind gift of Richard Needleman (15), was grown in light at 37°C in liquid growth medium containing neutralized peptone (L34, Oxoid), prepared as previously described (16).

PM isolation

PMs were isolated and purified on a sucrose density gradient as previously described (16).

Lipid extraction

18.75 ml of methanol-chloroform (2:1, v/v) was added to a PM suspension containing about 8 mg of BR in 5 ml of water. The mixture was gently shaken for several minutes until complete protein denaturation and bleaching was obtained. After centrifugation, the supernatant extract was decanted into a separatory funnel, and the residue was re-suspended in 23.75 ml of methanol-chloroform-water (2:1:0.8). The mixture was then shaken and centrifuged; 6.25 ml each of chloroform and water were then added to the combined supernatant extracts to obtain a two-phase system. After complete separation of the two phases (requiring a few hours at room temperature, in the dark), the chloroform phase, diluted with benzene, was brought to dryness under nitrogen; dried lipids were resuspended in a small chloroform volume and saved at −20°C. In order to verify that the lipid extraction was complete, the whitish denaturated BR (opsin) left after the lipid extraction was resuspended in methanol-chloroform-water (2:1:0.8), and residual lipids were reextracted following the above procedure.

Isolation and purification of individual lipid components of the PM

The lipids of PM were separated by TLC in solvent A (chloroform-methanol-90% acetic acid, 65:4:35, v/v/v). After scraping the silica in each band from the plate, lipids were extracted from the silica three times with chloroform-methanol-water 1:2:0.8 (v/v). After centrifugation, the supernatants were combined, and, by adding the appropriate amount of chloroform-water 1:1 (v/v), two phases were obtained. The chloroform layer was collected, diluted with benzene, and dried under a stream of nitrogen. Each component was further purified by re-chromatography in neutral solvent B (chloroform-methanol-water, 65:25:4, v/v/v) and recovered from silica as just described. The purity of final material was checked by means of silica gel 60A HPTLC in solvent A. The neutral lipids of PM were separated by TLC in solvent C (hexane-diethyl ether-acetic acid, 70:30:1, v/v/v); squalene was identified by comparison with an authentic standard.

NMR spectroscopy

^{31}P NMR analysis of phospholipids present in the PM lipid extract was performed by following the previously described method (17). The method is based on the use of a methanol reagent containing D_2O and a dissolved EDTA salt, prepared as follows. The cesium salt of EDTA was prepared by titrating a 0.2 M suspension of EDTA free acid with CsOH to a pH of 6.0, at which point free EDTA was in solution; EDTA salt solutions were evaporated to dryness on a freeze-dry apparatus, dissolved in a minimum volume of D_2O to exchange labile ^{1}H for ^{2}D, dried a second time, and dissolved in D_2O to a concentration of 0.2 M. The final methanol reagent, Cs/EDTA analytical reagent, was prepared by dissolving 1 ml of D_2O-EDTA solution in 4 ml of methanol. The cesium preparations were stable in sealed bottles. The D_2O was used solely in order to provide a deuterium reference signal for magnetic resonance field-frequency stabilization; it is not essential for signal narrowing. To prepare the sample, 1–5

mg of individual purified phospholipids or of total lipid extract was dissolved in 0.8 ml of deuterated chloroform. To this solution 0.4 ml of methanol reagent (containing Cs/EDTA) was added, and the mixture stirred gently. Two liquid phases were obtained, a major chloroform phase and a smaller water phase. By using a pasteur pipette, the sample was placed in a NMR test tube where it separated within 1 min. The sample tube turbine was adjusted so that only the chloroform phase was sensed by the NMR spectrometer's receiver coil. Magnetic field stabilization was obtained through the deuterium resonance of deuterated chloroform. Unless otherwise specified, samples were analyzed with proton broad-band decoupling to eliminate ^{1}H-^{31}P multiplets. Under these conditions, each spectral resonance corresponds to a single phosphorus.

^{1}H-NMR spectra were taken in $CDCl_3/CD_3OD$ (4:3 v/v); approximately 5 mg of total lipid extract dissolved in 700 μL were analyzed. All NMR analyses were performed on a DRX500 Avance Bruker instrument equipped with inverse probes for inverse detection and with z gradient for gradient-accelerated spectroscopy. ^{1}H chemical shifts are given relative to tetramethylsilane as an internal standard. ^{1}H decoupled ^{31}P chemical shifts are relative to 85% H_3PO_4 as an external standard. The spectra were recorded taking 64K data points for ^{1}H and 256K for ^{31}P, obtaining a digital resolution of approximately 0.2 Hz/point. In order to perform quantitative analyses, the flip angle was set to 45° and 75° for ^{1}H and ^{31}P acquisition, respectively, and the total time (acquisition plus recycle delay) between scans was selected in order to allow complete relaxation of nuclei. Moreover, the transmitter offset was set in the center of the spectral window, in proximity to the phospholipid resonances.

Inverse-detected ^{1}H-^{31}P-correlated 2D NMR spectra were obtained by using the standard gradient-enhanced pulse sequence INVIETGPSI, using a 7 Hz coupling constant for magnetization transfer (18–20).

RESULTS

TLC and electro-spray ionization mass spectrometry (ESI-MS) analyses have been used to gain preliminary insights into the lipid composition of the lipid extract of the PM isolated from *Halobacterium salinarum* cells. **Figure 2A** shows the TLC of the total lipid extract of the *Halobacterium salinarum* cells and of the lipid extract of the PM isolated from the same cells. As regards the PM lipids, besides neutral lipids (including retinal) at the solvent front, 6 lipid components are visualized on the plate. The abbreviated name of the individual lipids is reported at each spot site. Comparison of the two different lipid profiles in Fig. 2A (cell total lipids and PM lipids) is useful because it reveals that: 1) both the novel cardiolipins, GlyC and BPG, appear to be enriched in the PM lipid extract compared with the cell lipid extract, 2) the amount of PGS in the PM lipid extract is reduced compared with the cell total lipid extract, 3) the relative proportions of PG, PGP-Me, and S-TGD-1 are similar in the two different lipid profiles. These data suggest that the novel cardiolipins are specific components of the PM and, at the same time, raise doubts about the specificity of the location of S-TGD-1, which had been previously considered specifically located in the PM (3, 8). Further experiments are in progress to establish whether or not S-TGD-1 is a specific glycolipid of the PM. Figure 2B shows the ESI-MS spectrum of the lipid extract of

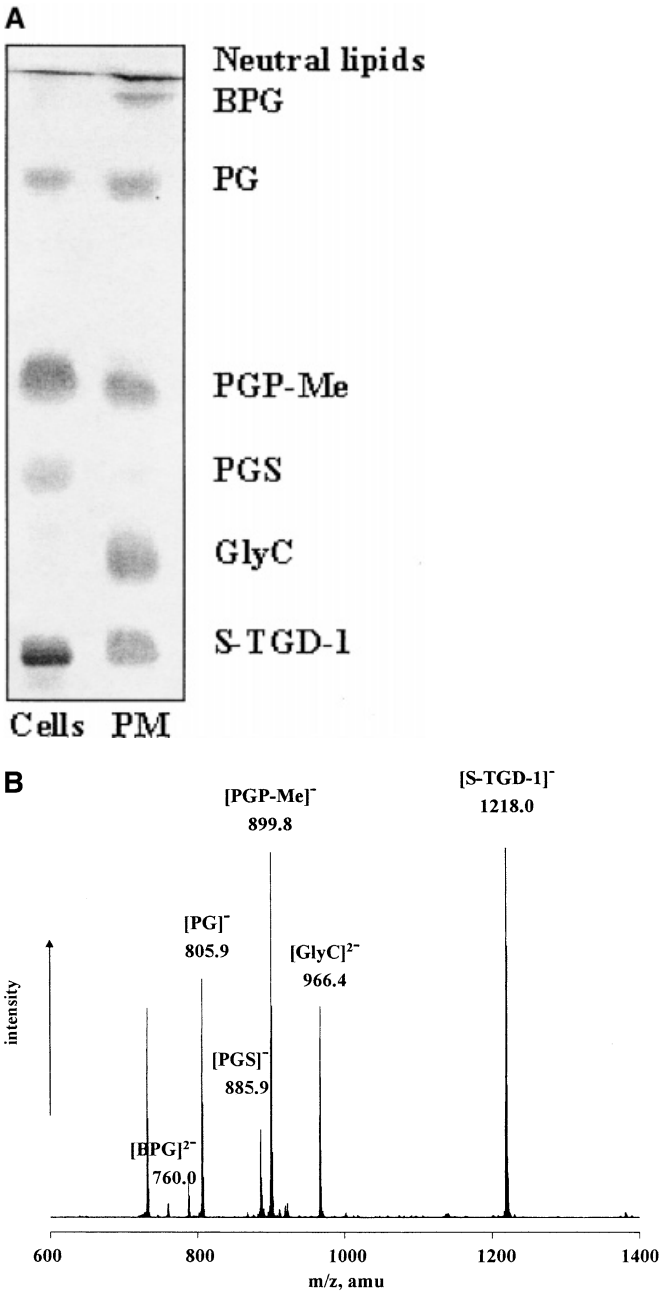

Fig. 2. TLC and electro-spray ionization mass spectrometry (ESI-MS) analyses of PM lipids. A: The chromatogram compares total polar lipids from halobacterial cells (cells) and total lipids of PM. B: Negative ion ESI-MS of total PM lipid extract.

the PM, including the molecular mass of negatively monocharged and bi-charged phospholipids and glycolipids.

Figure 3 shows the ^{31}P-NMR spectrum of the total lipid extract of the PM dissolved in the Cs/EDTA analytical reagent previously described in the literature (17). The use of the Cs/EDTA analytical reagent is necessary to reduce and avoid the broadening of signals of the phospholipids present in the crude lipid extract.

Only five of the TLC spots in Fig. 2A were also stained by molybdenum blue, staining specifically for the phospholipids: GlyC, PGS, PGP-Me, PG, and BPG. Among

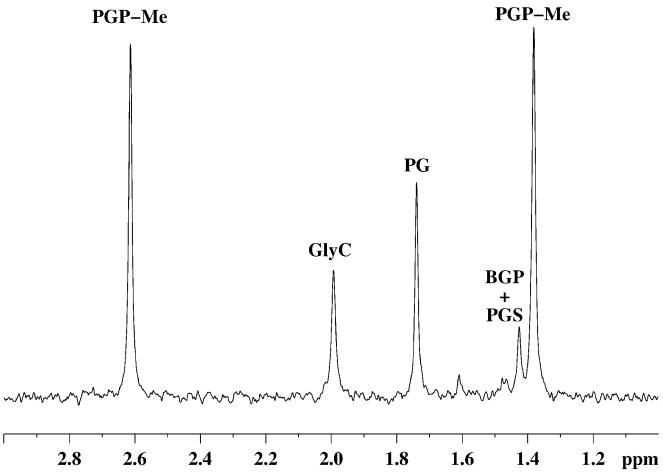

Fig. 3. ^{31}P-NMR of the total PM lipid extract.

these phospholipids there are two molecular structures containing two phosphate groups per molecule: PGP-Me and BPG. Whereas PGP-Me shows two distinct phosphorus signals when analyzed by ^{31}P-NMR spectroscopy, BPG exhibits only one sharp phosphorus peak, the two phosphorus atoms being chemically equivalent. Therefore, six different phosphorus signals are to be expected into the ^{31}P-NMR spectrum of the PM lipid extract. Four main peaks, plus one minor signal, are visible in Fig. 3. To assign peaks to individual PM lipids in Fig. 3, we analyzed each isolated and purified phospholipid of the PM by ^{31}P-NMR analysis.

Table 1 presents the ^{31}P-NMR chemical shifts obtained from isolated and purified archaeal phospholipids of the PM dissolved in the Cs/EDTA-containing reagent. By comparing the chemical shifts in the spectrum in Fig. 3 with those of the authentic standards in Table 1, it is possible to assign the major peaks to GlyC (1.96 ppm), PG (1.72 ppm) and PGP-Me (1.35 ppm and 2.59 ppm). The assignment of the two different phosphorus signals of PGP-Me was obtained by ^{31}P proton coupled NMR analysis (not shown). The multiplicity of the signal at 2.59 ppm is in agreement with the presence of the methyl phosphate, whereas the other resonances at 1.37 ppm have been attributed to the phosphate linked to C1 of glycerol. It appears quite difficult to distinguish PGS and cardiolipin in the spectrum in Fig. 3, because their resonances almost overlap (at 1.37 ppmand 1.4 ppm, respectively) and are very close to one of the two signals of PGP-Me (at 1.36 ppm). In order to check the identity of peak at 1.4 ppm, we analyzed by ^{31}P- NMR analysis an aliquot of the PM lipid extract enriched by a known amount of isolated and purified BPG. This analysis revealed that the addition of BPG to the extract increases the peak to 1.4 ppm. This last sample was also analyzed by inverse-detected heterocorrelated ^{1}H-^{31}P bidimensional NMR (see **Fig. 4A**). Such an experiment allows the identification of the different pro-

TABLE 1. ^{31}P chemical shifts of the individual purple membrane (PM) phospholipids

Lipid	^{31}P
	ppm
GlyC	1.964
PGS	1.374
PGP-Me	1.357 and 2.594
PG	1.725
BPG	1.401

Each phospholipid was isolated by TLC on solvent A and re-chromatographed on solvent B. Samples were prepared as described in methods. Individual PM phospholipids were isolated and purified as described in methods; approximately 2 mg of each phospholipid was solubilized in the Cs/EDTA-containing reagents.

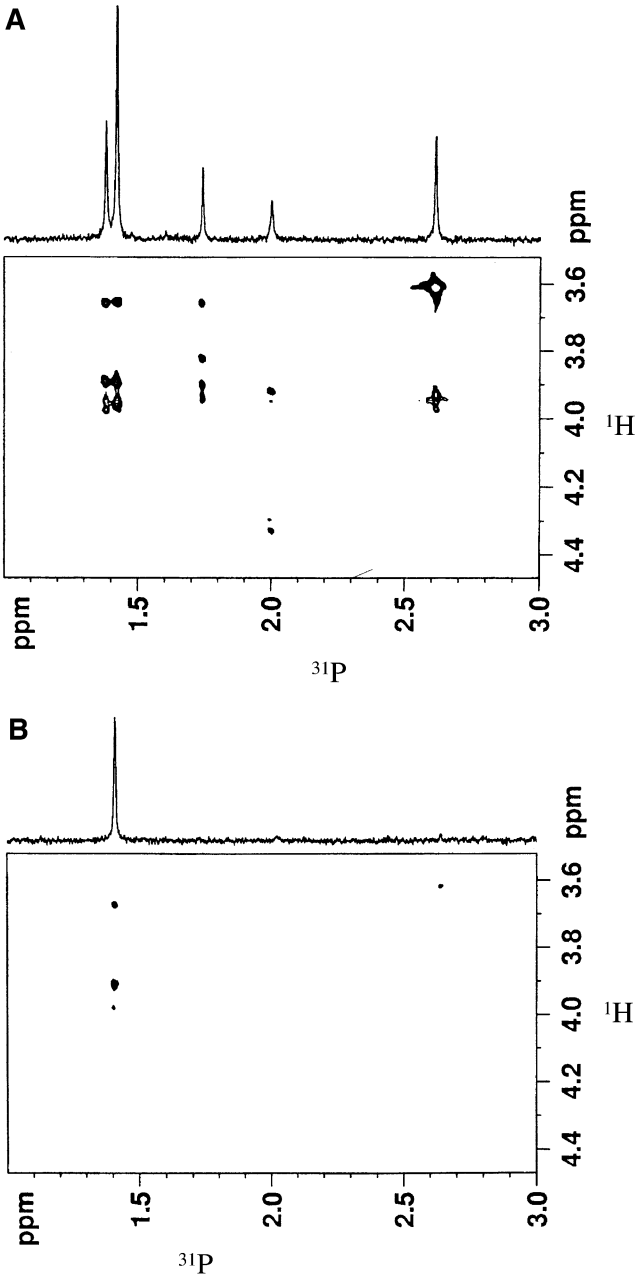

Fig. 4. Inverse-detected heterocorrelated ^{1}H-^{31}P bidimensional NMR of A: Sample of lipid extract (5 mg of lipids) containing an additional amount of archaeal cardiolipin (BPG) (1.8 mg), B: Isolated and purified phosphatidylglycerosulphate (PGS).

ton network coupled to each individual phosphorus. Similarities between BPG (major phosphorus peak due to BGP enrichment of the sample) and the C1 phosphorus-linked moiety of PGP-Me, together with differences with respect to other phosphorus environments, could be easily observed. On the other hand, the ^{1}H-^{31}P 2D spectrum (Fig. 4B) of the isolated and purified PGS, besides confirming its chemical structure, shows strict similarities with the 2D cross peaks pattern of BPG and of the C1 phosphorus linked moiety of PGP-Me. Because the patterns of PGS, BPG, and the C1-phosphorus-linked moiety of PGP-Me in the 2D spectra overlap, and as both PGS and BPG are present in much lower amounts compared with PGP-Me, an accurate quantitative analysis of both PGS and BPG in the lipid extract could not be carried out using 2D NMR spectra.

The proportions of the major lipids in the lipid extract were obtained by comparing the areas of the peaks in the ^{31}P NMR spectrum in Fig. 3. For the quantitative analysis, the ^{31}P peaks at 1.96 ppm, 1.72 ppm, and 1.36 ppm for GlyC, PG, and PGP-Me, respectively, were used, as they correspond to phosphorus having the same chemical environment. A rough estimation of PGS and BPG amounts in the extract was obtained by determining the area of the minor peak at 1.4 ppm, which was considered as the result of the sum of both PGS and GlyC signals. After deconvolution (Lorentz) of the peaks at 1.36 ppm and 1.4 ppm, we found that the molar ratio of PGS plus BPG to GlyC is 0.37 and that the molar ratio of PGP-Me to GlyC is 2.4. Furthermore, data in Fig. 3 indicate that the molar ratio of PG to GlyC is 1.2.

Further qualitative and quantitative data on lipids present in the total PM lipid extract was obtained by the proton NMR spectrum illustrated in **Fig. 5A**. Although there is a considerable overlapping of resonances from the different lipid components, some of the lipids have a structure-specific resonance or set of resonances that allow their rapid identification and quantitation.

Particularly in the 4.3–5.1-ppm region, there are twin signals attributable to the anomeric protons of the two glycolipids S-TGD-1 and GlyC, which both have the same three sugars in the same sequence in the polar head (see molecular structures in Fig. 1). In addition, the olefinic protons of squalene are also clearly distinct. The twin doublets around 4.4 ppm are characteristic for the anomeric proton of galactose but are partially masked by the water peak, whereas the doublets of anomeric protons of glucose (approximately 4.85 ppm) and mannose (approximately 4.80 ppm) are isolated from other signals (see the amplified interval in Fig. 5B). However, as the mannose anomeric protons of the two glycolipids are too close to be distinguishable, we used the glucose peaks as characteristic signals of the two glycolipids. At first sight, by looking at the twin signals of the anomeric protons of glucose, it is evident that more of one of the glycolipids is present than the other. By deconvolution (Lorentz) of peaks, the molar ratio of one glycolipid to the other was found to be 3.

In order to assign each of the twin anomeric signals unequivocally to the appropriate glycolipid, we analyzed the proton NMR spectrum of an aliquot of the PM lipid extract enriched with a known amount of authentic S-TGD-1. The amplified region 4.8–5.1 ppm of this spectrum is reported in Fig. 5B. By comparing the relative heights of the signals, it is clear that the addition of authentic S-TGD-1 to the extract resulted in the increase of the doublet at 4.87 ppm. As a consequence, the doublet at 4.85 ppm was assigned to the anomeric proton of GlyC.

From the data in Fig. 5B, it is therefore concluded that the molar ratio of S-TGD-1 to GlyC into the PM lipid extract is 3.

Because retinal is also present in the extract, proton NMR analyses allow a quick estimation of the molar ratios of individual lipids to retinal in the PM lipid extract. Assuming that lipid extraction is complete, this ratio is also representative of the molar ratio of lipid to BR into the PM. Considering that both *cis* and *trans* retinal isomers are present in the extract, the sum of the areas of cis and trans aldehydic proton signals at 10 ppm represents the total amount of retinal in the lipid extract. We found that the integral ratios of the characteristic signals of S-TGD-1 and GlyC to retinal are 3 and 1, respectively. As regards the neutral lipids, by integration of the olefinic multiplet of resonances at 5.1 ppm, the molar ratio of squalene to retinal appears to be 2, whereas from the weak benzylic resonances at 8 ppm, it is evident that vitamin MK-8 occurs as a negligible component compared with the other PM lipids. ^{1}H-NMR analysis of samples prepared by dissolving known amounts of the authentic standards S-TGD-1, squalene, and *all-trans* retinal showed that in our experimental conditions the relative areas of the characteristic proton resonances of the three different lipids are in agreement with the experimental molar ratios (not shown).

By combining data obtained by phosphorus and proton NMR analyses, the ratio of all major lipid components to retinal in the PM lipid extract can be obtained. For example, if the molar ratio of PGP-Me to GlyC is 2.4, and the molar ratio of GlyC to retinal is 1, it can be concluded that the molar ratio of PGP-Me to retinal is 2.4. **Table 2** reports the molar ratios of individual lipids (except for those of the minor components PGS and BPG) to retinal observed in the present study compared with previous data from Kates and coworkers (12).

DISCUSSION

Thirty years ago, the first estimation of the molar ratio of lipids to BR in the PM was obtained using the X-ray dimensions of the unit cell, the known proportion of lipid to protein, and the approximate molecular weight of the components. The calculations indicated that approximately 10 lipid molecules per BR molecule were present in the PM lattice (21).

Chemical analysis of the lipid extract of the PM was first carried out in 1975 (10). TLC analyses of the PM lipid extract allowed identification and quantification of PM lipids and indicated that the molar ratio of lipid to protein was approximately 7:1, thereby corresponding to approxi-

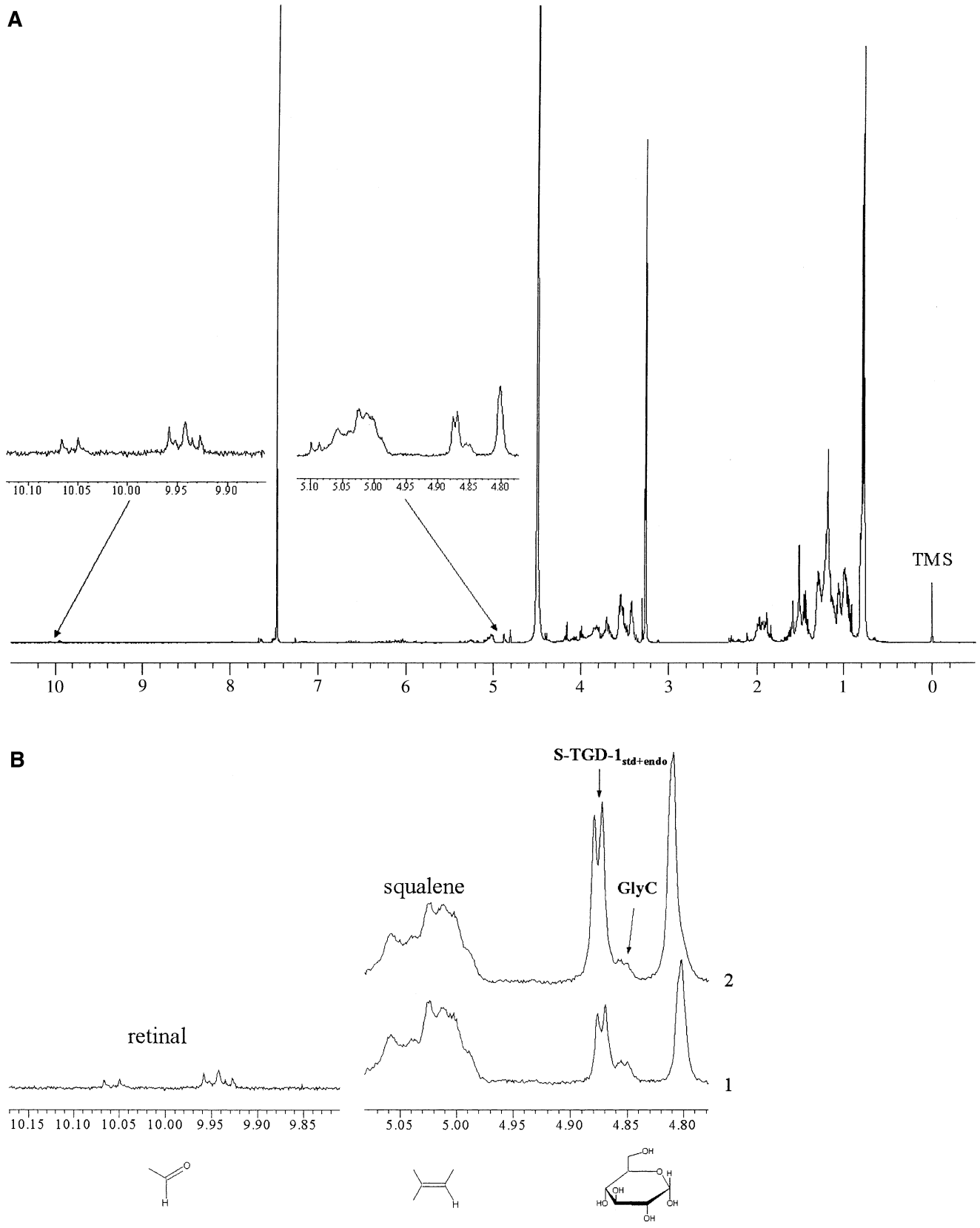

Fig. 5. A: ^{1}H-NMR of the total PM lipid extract (approximately 5 mg); the inserts enlarge the two regions around 10 and 5 ppm; peaks at 7.26, 4.5, and 3.3 ppm are due to residual protic solvents. B: Zoom of the 4–5.1 region of the ^{1}H-NMR spectrum, in the absence (1) and in the presence (2) of an additional amount of glycolipid sulfate (S-TGD-1) authentic standard; the region of the aldeidic protons of retinal is also shown.

TABLE 2. Lipid/retinal molar ratios in the total lipid extract of the PMs[a]

	NMR Data	Observed Nuclei	Kates et al. (12)
S-TGD-1	3	^{1}H	2
GlyC	1	^{1}H and ^{31}P	Missing
PGP-Me	2.4	^{31}P	3–4
PG	1.2	^{31}P	0.3
PGS + BPG	0.37	^{31}P	0.3[b]
Squalene	2	^{1}H	0.6
Vitamin MK-8	Traces	^{1}H	Traces
Total	10		7–8

[a] Data in the present paper (first column) are compared with previous literature data [see ref (12), third column].

[b] Refers only to PGS, as the cardiolipin BPG was not known at that time.

mately 21 molecules per unit cell. The polar lipids alone were found to amount to 6 molecules per BR molecule (or 18 molecules per unit cell). The major component of PM polar lipids was found to be the PGP-Me amounting to 3–4 molecules per BR molecule. The other polar lipids were S-TGD-1 (amounting to approximately 2 molecules per BR molecule), with PG and PGS as minor components (both amounting to 0.3 molecules per BR molecule). Neutral lipids (represented mainly by squalene) were found to be about 10% of total PM lipids, amounting to approximately 2 molecules per unit cell. Later the lipid composition of PM was reanalyzed by Dracheva et al. (22), obtaining similar results.

In this study we have obtained novel data on the lipid composition of the PM by combining ^{31}P-NMR and ^{1}H-NMR analyses, for the first time also taking into account two novel cardiolipin analogs, recently found in the PM (13). The various components of the PM lipid extract could be identified by specific characteristic resonances in the proton and phosphorus NMR spectra. Employing appropriate calibration procedures, the technique adopted here provided accurate mole-fraction data on the main phospholipids of the PM. Furthermore, taking advantage of the fact that retinal is also completely removed together with membrane lipids during the extraction procedure, we have also obtained for the first time a direct estimation of lipid/BR stoichiometries in the PM.

The data in the present article indicate that in total, 10 lipids per BR are present in the PM. Of these, five are phospholipids, three are glycolipid sulfate, and two are squalene. Among the phospholipids, as regards the cardiolipin analogs, one GlyC per BR was found in the PM, while the other cardiolipin was a minor component.

We have shown in previous studies that the novel archaeal analogs of cardiolipin of the PMs are tightly bound to BR and appear to have a key role in preserving the stability of solubilized BR, in particular the trimer aggregates (23). As a consequence of this experimental evidence, a question arises: Are the novel cardiolipins located inside BR trimers? Although the precise location of lipids in the PM lattice has not yet been determined, some evidence regarding the glycolipid location in PM is available in the literature. It is well known that glycolipids are specifically located on the PM extracellular face (24). Recent elegant neutron diffraction studies of PM containing deuterium-labeled S-TGD-1 have shown density at two locations per BR monomer, in the inter- and intra-trimer space (25). Specific interactions of bacteriorhodopsin with endogenous glycolipids have also been described in detail in BR crystals (1). Three glycolipids have been found to face the extracellular side in the central compartment of BR trimers in monoclinic BR crystals, underlining their important role for intra-trimer stabilization (1). The molar ratio of GlyC to BR in the purple membrane appears to be consistent with its possible location in the intra-trimer space. Such a location for three GlyC would also be consistent with the previous identification of 12 well-defined side chains in the central trimer compartment (1). Interestingly, eukaryal cardiolipins have been identified and localized in both crystals of the photosynthetic reaction center of *Rhodobacter sphaeroides* (26, 27). Furthermore, tightly bound cardiolipin was shown to have a role in stabilizing the quaternary structure of cythocrome bc1 (28).

Finally, consistent with its low amounts relative to retinal in PM (BPG/retinal <0.37), we can speculate that the minor phospholipid BPG could be specifically located in the inter-trimer spaces, and that it could mediate specific interactions between trimers by establishing bridging contacts between BR molecules in adjacent trimers in the PM lattice. An inter-trimer location may also be suggested for the other minor phospholipid PGS, which is capable of reestablishing BR trimers into an hexagonal array in reconstituted systems (29).

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