Lateral Proton Conduction in Monolayers of Phospholipids from Extreme Halophiles†

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ABSTRACT: Studies have been carried out on the lateral proton conductance properties of monolayers of the major and minor phospholipids of extremely halophilic archaebacteria, 2,3-diphytanyl-*sn*-glycero-1-phospho-3′-*sn*-glycerol 1′-phosphate (PGP) and 2,3-diphytanyl-*sn*-glycero-1-phospho-3′-*sn*-glycerol (PG), respectively, as well as on their respective deoxy analogues: 2,3-diphytanyl-*sn*-glycero-1-phospho-1′-propanediol 3′-phosphate (dPGP), 2,3-diphytanyl-*sn*-glycero-1-phospho-1′-1′,3′-propanediol (dPG), and 2,3-diphytanyl-*sn*-glycero-1-phospho-1′-propanol (ddPG). Lateral proton conduction was found to occur with monolayers of all ether phospholipids examined at reduced surface pressure ($\pi > 25$ mN/m) on subphases of low (1 mM) and high (4 M) ionic strength. Proton conduction was also detected in highly condensed monolayers (>35 mN/m) of the naturally occurring phospholipids (PGP, PG) but was abruptly terminated in tightly packed monolayers (>35 mN/m) of the corresponding deoxy compounds (dPGP, dPG, ddPG) on subphases with low ionic strength. Conduction did occur, however, along monolayers of the deoxy compounds at high surface pressure when spread on a subphase of high ionic strength (4 M). The abrupt termination of conduction with monolayers of the deoxy compounds at low ionic strength cannot be attributed to a lipid phase transition or to changes in the lateral fluidity of the monolayers, nor was the p*K* of the fluorescent interfacial proton indicator affected at high surface pressures. Our data are consistent with the occurrence of a conformational change in the polar headgroup region of the deoxy compounds under high compression of the monolayers but not in that of the naturally occurring phospholipids. The most likely change in the polar headgroup of the deoxy compounds would be the formation of internal hydrogen bonding only under high surface pressure, which would expel water molecules and result in disruption of the conducting network. In the natural phospholipids PGP and PG, the presence of the central hydroxyl group would facilitate the formation of stable intramolecular bondings which would render the molecular structure of their headgroups insensitive to high surface pressure and hence preserve the proton conduction network.

The extremely halophilic archaebacteria are distinguished from other archaebacteria and from eubacteria by the presence of two distinct energy transducing membranes, the purple membrane which functions as a light-activated proton pump and the red membrane which acts as a proton-consuming ATP synthetase (Stoeckenius, 1980). The coupling between these two systems needs to be very effective in order for the bacteria to utilize the light-generated proton gradient for ATP synthesis. Experiments with chloroplasts (Auslander & Junge, 1974) and with *Escherichia coli* (Gould & Cramer, 1977), in which phosphorylation was observed before proton ejection in the bulk phase was detected, suggested a direct proton coupling. The existence of a lateral membrane localized proton transfer between the proton pump and sink has been demonstrated by direct experiments with phospholipid monolayers at the air/water interface using both fluorescence and surface potential techniques (Teissié et al., 1985; Prats et al., 1986). This proton conduction was shown to occur with a variety of phospholipids but was a function of the nature of the polar headgroups (Prats et al., 1987). These results led to the conclusion that proton conduction occurs along a hydrogen-bonded network consisting of the polar headgroups and the water molecules of the hydration layer. Such a mechanism is in agreement with that proposed 20 years ago to explain the electrical conductivity of phospholipid bilayers (Leslie et al., 1967) and more recently of phospholipid monolayers (Sakurai & Kawamura, 1987).

The red and purple membranes of extremely halophilic archaebacteria contain unusual diphytanyl ether linked phospholipids and glycolipids: 2,3-diphytanyl-*sn*-glycero-1-phospho-3′-*sn*-glycerol 1′-phosphate (PGP),[1] 2,3-diphytanyl-*sn*-glycero-3′-phospho-1-*sn*-glycerol (PG), and 1-(3-sulfonatogalactosyl-β-1–6-mannosyl-α-1–2-glucosyl-α-1–1)-2,3-diphytanyl-*sn*-glycerol (STGD) (Figure 1) (Kates, 1978; Kamekura & Kates, 1988).

The red and purple membranes have characteristic distributions of these species of lipids: the purple membrane contains both STGD and PGP in the outer leaflet but only PGP and PG in the inner leaflet (Henderson et al., 1978; Kates et al., 1982; Kamekura & Kates, 1988), while the red membrane contains mainly PGP in both leaflets (Kushwaha et al., 1975; Kamekura & Kates, 1988).

It was of interest then to study the proton conductance properties of these major individual lipids to determine whether they could function in a localized energy transducing pathway.

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[1] Abbreviations: PGP, 2,3-diphytanyl-*sn*-glycero-1-phospho-3′-*sn*-glycerol 1′-phosphate; PG, 2,3-diphytanyl-*sn*-glycero-1-phospho-3′-*sn*-glycerol; dPGP, 2,3-diphytanyl-*sn*-glycero-1-phospho-1′-propanediol 3′-phosphate; dPG, 2,3-diphytanyl-*sn*-glycerol-1-phospho-1′-1′,3′-propanediol; ddPG, 2,3-diphytanyl-*sn*-glycero-1-phospho-1′-propanol; STGD, 1-(3-sulfonatogalactosyl-β-1–6-mannosyl-α-1–2-glucosyl-α-1–1)-2,3-diphytanyl-*sn*-glycerol; FPE, fluorescein phosphatidylethanolamine thiocarbamide; TPL, total polar lipids of *H. cutirubrum*; FRAP, fluorescence recovery after photobleaching.

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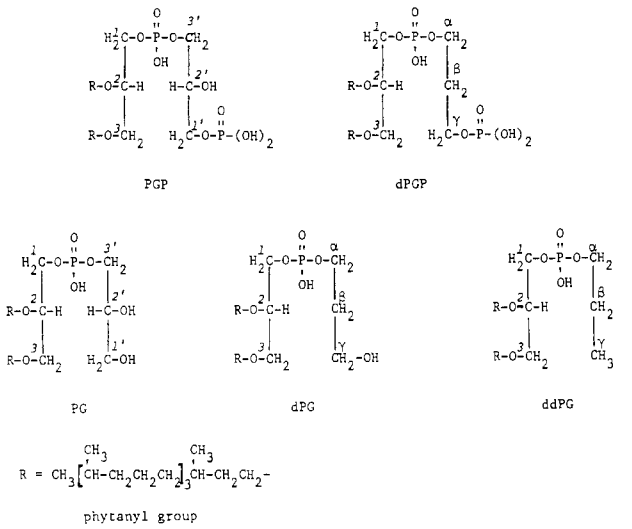

FIGURE 1: Structures of diphytanyl phospholipids and their deoxy analogues.

Such a function would be in line with the observed optimal proton pumping of bacteriorhodopsin when reconstituted in lipid vesicles of PGP and STGD (Hojeberg et al., 1982). In order to probe the structure–function relationship of PG and PGP in proton conductance on a molecular level, we have taken advantage of the availability of deoxy analogues of these lipids (dPG and dPGP) (Figure 1) in which the central free hydroxyl group of the glycerol residue has been replaced by a hydrogen atom. Recent studies have shown that this central hydroxyl group plays a critical role in both intra- and intermolecular hydrogen bonding and in the associated hydration with interfacial water (Stewart et al., 1988, 1989).

The present results show clearly that the naturally occurring halophile membrane lipids (PGP and PG) are well suited to function in a lateral proton conducting system.

Materials and Methods

Chemicals. Total polar lipids (TPL), STGD, PG, and PGP were isolated from cells of *Halobacterium cutirubrum* as described elsewhere (Kates, 1978). The deoxy analogues (dPG, dPGP, and ddPG) (Figure 1) were synthesized chemically (Stewart et al., 1988). Synthesis of the fluorescent pH indicator probe fluorescein phosphatidylethanolamine thiocarbamide (FPE) was described previously (Soucaille et al., 1988). 12-(9-Anthroyloxy)stearic acid was purchased from Sigma. Salts were analytical grade. Ultrapure water free from surfactant was prepared with a Milli-Q system (Millipore, France).

Monolayer Preparation. Phosphate- (1 mM) buffered saline solutions were prepared with ultrapure water. Lipids were spread from solution in chloroform/methanol (9:1 v/v), and measurements were made after a 5-min period to allow for solvent evaporation. The film surface pressure was monitored by means of a platinum plate (Prolabo, France) connected to a force transducer of our own construction. The sensitivity of the surface pressure determination exceeded 0.2 mN/m.

Fluorescence Measurements. An interface fluorometer constructed in the laboratory was used, in which the front-face fluorescence from a small illuminated area (about 2 mm in radius) was monitored for different compression states of the monolayer (Teissié et al., 1976; Teissié, 1979). The trough (volume 100 mL) was milled in Plexiglas in order to maintain a low degree of light scattering. The film pressure was measured by a platinum plate. Compression was obtained by moving a Teflon barrier in order to change the total surface area of the monolayer (always less than 100 cm^2). Excitation wavelengths were selected by means of optical filters. The fluorescence intensity was measured by means of a high-sensitivity photomultiplier tube (EMI 9558, England) connected to a data acquisition unit.

Photobleaching experiments were carried out as follows. The background signal, due to the light scattered by the subphase, was set at zero fluorescence electronically. Then a mixture of probe [2-(9-anthroyloxy)stearic acid] and phospholipid (2:98 molar ratio) (about 10^{15} molecules) in an organic solvent (chloroform/methanol, 9:1) (10 μL) was spread on the surface at 21 °C in the dark to avoid photodegradation at the air/water interface. After an equilibration period of 3 min to allow for solvent elimination, the film was then compressed to a given pressure and allowed to equilibrate for an additional 5 min. The optical shutter was then opened, and the decrease in fluorescence intensity resulting from the dimerization reaction was recorded. After a 30-s bleaching period, the shutter was closed, and recovery was detected by observing the fluorescence signal following irradiation of the monolayer for 1 s. The film was then compressed to a new value of the surface pressure, and the bleaching procedure was repeated.

As the surface concentration of the probe increased with compression of the monolayer, the intensity was expressd as the "reduced intensity" I_f, which is proportional to the fluorescence emitted by each probe molecule. We have previously shown that the reduced intensity is obtained as the product of the observed intensity times the molecular area of the lipid matrix (for a given probe to lipid molar ratio) (Teissié et al., 1976; Theretz et al., 1984). I_f is a linear function of the quantum yield of the chromophore and is dependent on the nature of the environment (Franck–Condon effect).

The dimerization reaction of 12-(9-anthroyloxy)stearic acid is second order with respect to the probe. As the dimer is nonfluorescent and as the fluorescence intensity of the monomer is linearly related to its concentration, the extent of reaction is obtained from the decay in fluorescence emission during illumination. The rate constant of dimerization K_d is obtained by plotting the reciprocal of fluorescence intensity versus time of illumination. This is in fact computed during the early steps of the photoreaction to obtain initial rates. In control experiments, no deviation from linearity, reflecting the occurrence of a recovery process during the bleaching, was detected. As described in our previous work (Theretz et al., 1984), the dimerization constant was shown to be a function of spectroscopic and structural contributions. The former are direct functions of the reduced fluorescence intensity I_f, and the latter reflect the influence of both the structure and the dynamics of the probe environment.

After photoreaction, the local concentration of monomers in the previously illuminated area is lower than that in the nonbleached surface. This concentration gradient then drives the diffusion of fluorescent monomers into the bleached zone. The extent of recovery of fluorescent monomers is a direct function of the lateral diffusion coefficient D of the probe (Teissié et al., 1978). Recovery experiments were analyzed by use of a mathematical approach adapted for uniform disk illumination (Teissié et al., 1978). In order to improve the reliability of this treatment, the experimental data were subjected to statistical analysis, taking into account the nonlinear relationship between extent of recovery and duration of recovery (Denicourt et al., 1987).

Proton lateral diffusion experiments were run with the proton "window" jump technique using a trough and an ex-

perimental procedure described previously (Teissié et al., 1985; Prats et al., 1985). Monolayers were obtained by spreading a mixture of phospholipids and FPE (molar ratio 98:2) in solution in $CHCl_3$/MeOH (5:1 v:v) onto an aqueous subphase (10 mM phosphate buffer at a well-defined pH). The movement of protons from the injection compartment to the fluorescence observation area was observed by a change of the fluorescence emission of the pH-sensitive fluorescent chromophore (FPE at the lipid/water interface). This proton lateral diffusion is described by two parameters: T_{H^+}, the time between acid injection and the beginning of the decrease in fluorescence; ΔF, the amplitude of this decrease (Teissié et al., 1985).

Determination of the Apparent pK (pK_{app}). pK_{app} was taken as the subphase pH at which the fluorescence F emitted by the film for a given surface pressure π obeyed the relationship

$$\frac{F(pK_{app},\pi) - F(\text{pH } 4,\pi)}{F(\text{pH } 7.5,\pi) - F(pK_{app},\pi)} = 1$$

the indicated pHs being those of the subphase. This operational definition was based on the observation that the fluorescence was not related in any obvious way to the subphase pH for values of pH outside the range 4.5–7.2. It was obtained by compressing films on subphases with different values of pH and recording the fluorescence intensity and the surface pressure as functions of the molecular area (Soucaille et al., 1988).

Results

Compression Isotherms. Monolayers of the different lipids were compressed, and the change in surface pressure was observed as a function of the molecular area. Results are shown in panels A–D of Figure 2 for PG, dPG, PGP, and dPGP, respectively. A small but significant expansion of the films of the deoxy derivatives, dPG and dPGP, was observed compared to the respective natural lipids, PG and PGP. No break in any of the compression isotherms was detected, indicating that no phase transition was induced upon compression over the range 0–40 mN/m.

The compression isotherms of the deoxy compounds were strongly affected by the ionic content of the subphase. When spread on a 4 M NaCl subphase, the films of the deoxy derivatives, dPG and dPGP, were largely expanded as compared to their behavior on a subphase of low ionic content (1 mM) (Figure 2B,D, curves a and b). In contrast, the compression isotherms of the natural compounds, PG and PGP, were relatively unaffected by the ionic content of the subphase (Figure 2A,C, curves a and b).

Fluorescence Changes of FPE upon Film Compression. The reduced fluorescence intensity, I_f, of the pH-sensitive probe, FPE, when embedded in a film of dPG at a low molar ratio (2%), was observed to be affected by compression of the monolayer (Figure 2). When dPG or PGP was spread on a subphase of low ionic content (1 mM), the plot of I_f versus host film molecular area showed a break at 1 nm^2 with a very strong decrease when the lipid matrix was tightly packed (Figure 2B,C, curve c). Such behavior has already been observed with different ester-linked phospholipids (Soucaille et al., 1988). When the compression isotherms were run with a subphase containing a high ionic content (4 M NaCl), a linear decrease in I_f was always present upon compression but at a lower level than that with a low ionic content subphase for dPG and a higher level for PGP (Figure 2B,C, curve d).

A linear decrease in I_f versus molecular area was observed for natural PG and the deoxy derivative, dPGP, on both 1 mM and 4 M NaCl subphases but at a lower level on 4 M NaCl with PG and a higher level on 4 M NaCl with dPGP (Figure 2A,D, curves c and d).

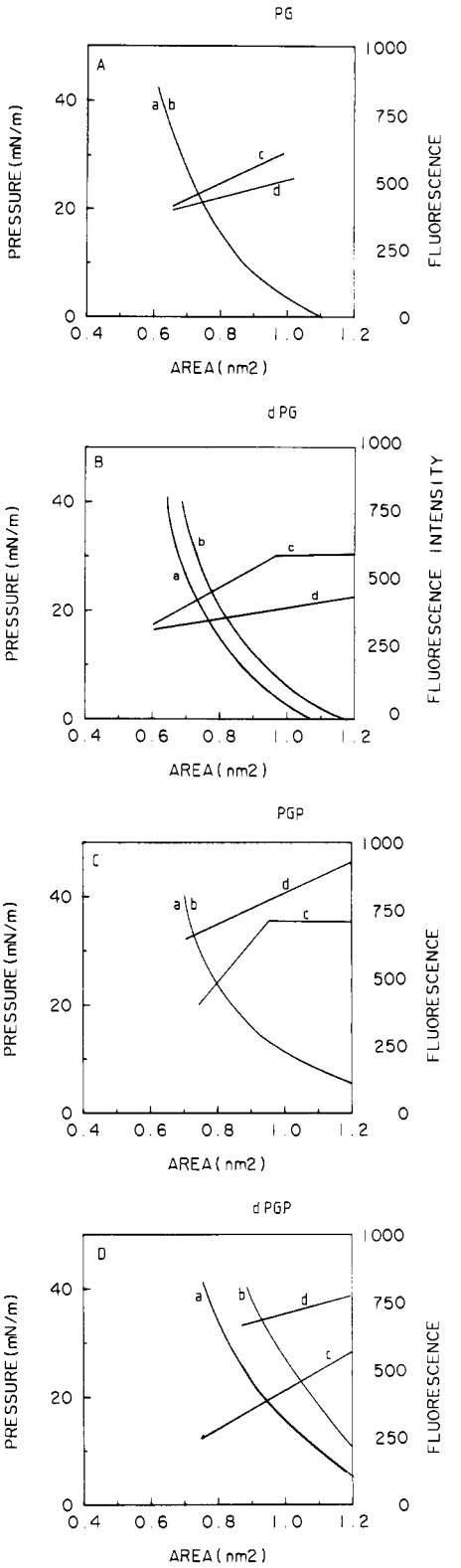

FIGURE 2: Compression isotherms of the diphytanyl phospholipids and the associated reduced fluorescence intensity changes of FPE embedded in the lipid matrix: (A) PG; (B) dPG; (C) PGP; (D) dPGP. The subphase contained a low ionic content (1 mM): (a) compression isotherm; (c) fluorescence. The subphase contained 4 M NaCl: (b) compression isotherm; (d) fluorescence. The probe to lipid molar ratio was 2:98.

As shown in Table I, the pK_{app} of the probe was only moderately affected by experimental conditions such as the

Table I: Values of Apparent pK (pK_{app}) of FPE Embedded in Various Diphytanyl Phospholipid Monolayers[a]

lipid	pK_{app}
dPG	6.0 (±0.1)
dPGP	5.8 (±0.1)
ddPG	6.2 (±0.1)
TPL	6.6 (±0.1)

[a] Monolayers were spread on a subphase of a low ionic content (1 mM) at a surface pressure of 20 mN/m; mole ratio of probe to host phospholipid is 2:98.

nature of the polar headgroup, ionic content of the subphase, and surface pressure of the film. These observations are consistent with our previous findings using monolayers of ester-linked phospholipids (Soucaille et al., 1988) and provide further evidence of the reliability of FPE as a pH indicator in the analysis of proton fluxes close to the membrane/water interface layer.

Lateral Proton Conduction along the Monolayer. When the lipids were spread on a low ionic subphase, very fast lateral proton conduction was always detected with the natural compounds (PGP, PG, STGD, and TPL) (Table II). T_{H^+} was always in the range of 2–4 min, and ΔF was not strongly affected by compression of the film. These results are very similar to those observed previously with ester-linked phospholipids (Teissié et al., 1985; Prats et al., 1987). It should be noted that, under our experimental conditions, if no lipid film is present, then T_{H^+} (detected by the fluorescence change of the water-soluble pH indicator fluorescein isothiocyanate) is very long (close to 1 h) (Teissié et al., 1985).

Under the same subphase conditions, a very striking result is observed when one works with the deoxy derivatives. Fast lateral conduction is no longer present when the film is highly compressed (pressure over 35 mN/m; area 0.65 nm^2) (Table II and Figure 3). This observation is valid for the three deoxy compounds which were tested (dPG, dPGP, ddPG). as shown in Figure 3, termination of the proton lateral conduction is a very dramatic process and occurs at a critical surface pressure (π = 25 mN/m for ddPG; π = 35 mN/m for dPG and dPGP). As reported above, no significant break in the compression isotherm was detected at these critical surface pressures.

When the experiments were performed with films spread on subphases containing a high ionic content (4 M NaCl or KCl), the fast proton lateral conduction was detected at all pressures (Table II).

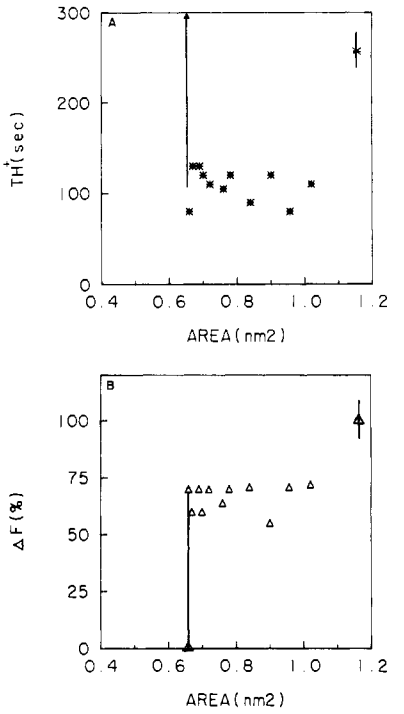

FIGURE 3: Changes in the lateral proton conduction parameters as a function of the packing of a dPG monolayer. The monolayer was spread on a low ionic content subphase. Parameters were obtained from the fluorescence changes of FPE embedded in the host lipid. (A) is T_{H^+}, and (B) is ΔF. The error bars are given in the upper right corners.

Structural and Dynamic Investigations of the Films. Previous studies on the lateral conduction of protons along phospholipid monolayers showed that the process was abolished when the film was compressed to the solid condensed state (Prats et al., 1987). If such a phase change also occured in films of the ether-linked phospholipids and their deoxy derivatives, it would provide a trivial explanation for the termination of the proton conduction just described. The photochemistry of 12-(9-anthroyloxy)stearic acid has been shown to be a powerful tool for the investigation of such phase transitions (Teissié et al., 1978; Denicourt et al., 1987). When

Table II: Lateral Proton Conduction along Diphytanyl Phospholipid Monolayers[a]

	pressure (mN/m)							
	5		15		25		35	
lipid	T_{H^+} (s)	ΔF (%)	T_{H^+} (s)	ΔF (%)	T_{H^+} (s)	ΔF (%)	T_{H^+} (s)	ΔF (%)
Spread on Low Ionic Content Subphase (1 mM Phosphate Buffer, pH 6.8)								
PG	200 ± 20	47 ± 5	165 ± 20	64 ± 8	165 ± 20	46 ± 8	205 ± 35	92 ± 8
dPG	200 ± 20	60 ± 5	195 ± 20	53 ± 5	195 ± 20	41 ± 3	*b*	*b*
PGP	225 ± 75	65 ± 5	165 ± 30	64 ± 9	240 ± 30	36 ± 5	285 ± 35	48 ± 10
dPGP	150 ± 10	89 ± 5	120 ± 20	63 ± 5	210 ± 30	35 ± 5	*b*	*b*
ddPG	300 ± 60	54 ± 5	145 ± 5	68 ± 6	*b*	*b*	*b*	*b*
STGD	140 ± 10	94 ± 5	145 ± 10	76 ± 5	170 ± 10	52 ± 7	245 ± 10	63 ± 12
TPL	150 ± 10	53 ± 4	120 ± 40	62 ± 5	150 ± 10	55 ± 5	nd	nd
4 M NaCl Subphase[c]								
PG	200	100	240	63	270	38	220	87
dPG	180	63	210	77	180	74	185	75
dPGP	210	89	180	88	180	79	200	90
ddPG	200	85	180	71	190	82	210	91
4 M KCl Subphase[c]								
PG	180	94	220	84	210	61	240	62
dPG	200	70	180	70	200	71	190	87

[a] Values are given as means ± SD. [b] No lateral proton conduction was observed. [c] The experimental deviation for T_{H^+} was ±20 and for F was ±10 under these conditions.

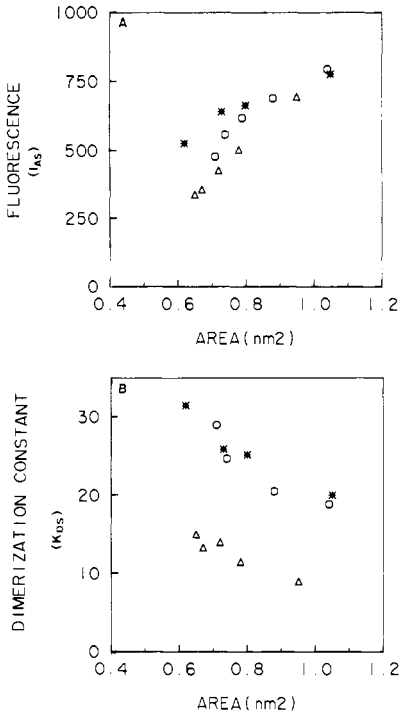

FIGURE 4: Photochemical parameter changes of 12-9-AS embedded in a dPG monolayer as a function of the host lipid molecular area. The fluorescent fatty acid was present at a low molar ratio (1.1%). The monolayer was spread on a low ionic content (1 mM) subphase (*) or on a 4 M NaCl subphase (O). (A) is the 12-9-AS reduced fluorescence intensity (obtained at zero time of photobleaching), and (B) is the structural dimerization constant K_{DS}. (Δ) are results for PG on a low ionic content (1 mM) subphase. The error bars are smaller than the size of the symbols.

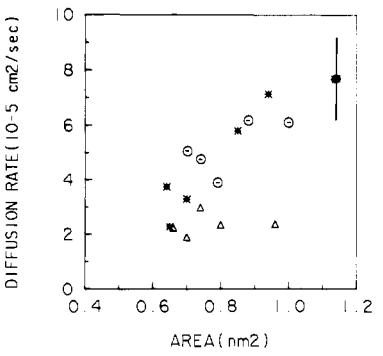

FIGURE 5: Changes in lateral diffusion coefficient D of 12-9-AS when embedded in a lipid monolayer. The probe was present at a low molar ratio (1.1%). D was calculated from the FRAP results. The host matrix was PG on a low ionic content subphase (Δ), dPG on a low ionic content subphase (*), and dPG on a 4 M NaCl subphase (⊙). The error bar is given in the upper right corner.

embedded in a lipid monolayer at a low molar ratio, this fluorescent fatty acid is an indicator of the structure and the order of the matrix. Its reduced fluorescence $I_{f^{12}AS^{9}}$ is indicative of the environment at the level of C_{12} of the fatty chains, its structural dimerization constant K_{DS} is an indicator of the organization of the matrix at the level of C_{12}, and the diffusion coefficient D (obtained from the FRAP experiments) is under the control of the order parameter of the matrix, the so-called "fluidity".

Results are shown in Figures 4 and 5 for dPG and PG. Similar results were obtained with PGP, dPGP, and STGD (data not shown). $I_{f^{12}AS^{9}}$ is observed to increase and K_{DS} to decrease upon compression as we reported for ester-linked phospholipids in the liquid-expanded state (Denicourt et al., 1987). The diffusion coefficient, D, decreases slightly upon compression, reflecting a small increase in the order parameter, as already described for ester-linked phospholipids in the liquid-expanded state (Peters & Beck, 1983). The same results were observed with dPG independent of the composition of the subphase (Figure 5), and it should be noted that when one compares the results obtained on a low ionic content subphase, the diffusion coefficient, D, is smaller with PG than with the deoxy derivative dPG at all surface pressures. Of course, this occurred when lateral proton conduction was present for PG but not for dPG.

All these observations provide direct experimental evidence showing that under the conditions used all of the phospholipids studied were always in the liquid-expanded state and that a liquid-condensed or solid-condensed state was not triggered upon compression. This finding is consistent with the fact that natural PGP, PG, and STGD form bilayers in aqueous dispersions that are in the liquid-crystalline state in the temperature range −35 to 80 °C, as expected for lipids containing the highly branched phytanyl groups (Chen et al., 1974).

Discussion

The present results show that lateral proton conduction occurs with all of the ether lipid analogues examined at reduced surface pressure on subphases of both high and low ionic strength (Table II). With the naturaly occurring compounds (PGP, PG, STGD), conduction is detected even in highly condensed monolayers (35 mN/m), in contrast to its abrupt termination in tightly packed monolayers of the corresponding deoxy compounds (dPGP, dPG, ddPG) (Table II and Figure 3). This effect was even more pronounced with monolayers of ddPG where no proton conduction was observed at a surface pressure as low as 25 mN/m. However, conduction did occur in monolayers of the deoxy compounds at high surface pressure when spread on a subphase of high salt content (4 M) (Table II).

It should be noted that the disappearance of conduction upon compression of monolayers of deoxy compounds spread on a low ionic subphase occurs abruptly without any intermediate decrease (Figure 3). This abrupt change in conduction cannot be explained by a change in pK of the proton probe (FPE) (Table I) nor to an abrupt change in fluidity of the lipid matrix as measured by FRAP (Figure 5). This is in contrast to previous observations with monolayers of dipalmitoylglycerophosphocholine (DPPC) which showed a change in conductance during compression through the liquid-condensed state (Prats et al., 1987). The dramatic termination of conduction observed with the deoxy compounds must therefore be associated with subtle changes in the monolayer organization which do not dramatically affect the compression isotherms (Figure 2). The results of the dimerization experiments (Figure 4) show that the abrupt change in proton conduction was not accompanied by changes in the organization of the hydrophobic region.

Thus, our experimental data are consistent with the occurrence of an alteration in the polar headgroup region of the deoxy compounds but not in the corresponding region of the naturally occurring phospholipids. It was shown previously that lateral proton transfer is localized in the polar headgroup region (Prats et al., 1986). This lateral transfer was explained by a "hop and turn" mechanism in the hydrogen-bond network formed by the hydrated polar headgroups (Prats et al., 1987), involving interactions between interfacial water and the polar moieties. The formation of hydrogen bonds is dependent on the presence of acceptor and donor groups (such as hydroxyl and phosphate groups) and on a precise distance fit between them.

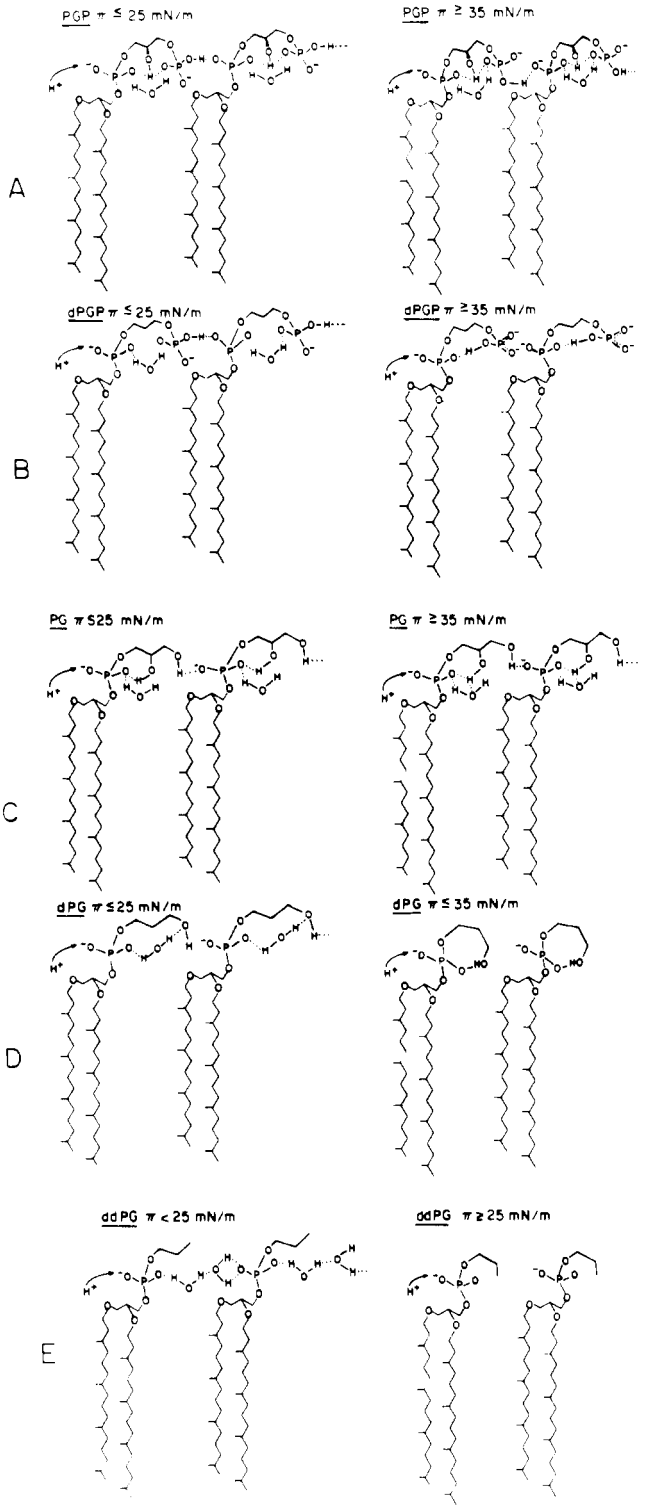

FIGURE 6: Conformational changes of the polar headgroup of the natural phytanyl phospholipids and their deoxy analogues upon external constraint: (A) PGP; (B) dPGP; (C) PG; (D) dPG; (E) ddPG. Conformations are shown for monolayers at low surface pressures (left; $\pi < 25$ mN/m) and at high surface pressures (right; $\pi > 35$ mN/m).

As can be seen in Figure 6A,C, the natural phospholipids (PGP, PG) can readily form intramolecular hydrogen bonds between the central hydroxyl of glycerol and the phosphate groups, as well as intermolecular hydrogen bonds between phosphate groups and external hydrogen-bond systems involving interfacial water. Formation of internal hydrogen bonding in PGP would be strongly facilitated by the fact that ionization of the third phosphate OH group ($pK = 11$) does not occur below pH 7 (Stewart et al., 1988). The molecular structure of PGP and PG would thus be stabilized by such an intramolecular hydrogen-bond system and would be rendered insensitive to external pressure (panels A and C of Figure 6, respectively). Recent Fourier transform infrared studies have provided evidence for such intramolecular hydrogen bonding in PGP but not in dPGP or in dPG (Stewart et al., 1989).

In contrast, dPGP and dPG could form a stable internal hydrogen bond only under an external constraint, necessary to obtain the right distance fit between the phosphate groups in the case of dPGP or between phosphate and the free hydroxyl group of dPG (panels B and D of Figure 6, respectively). With ddPG, no internal hydrogen bonding is possible (Figure 6E). The first two deoxy compounds can however form external hydrogen bonds with interfacial water but at the expense of the internal hydrogen bonds, under low surface pressure conditions. At high surface pressure, the induced internal hydrogen bonding would expel water molecules from the polar headgroup region and thus destroy the conducting network (Figure 6B,D). In the headgroup of ddPG, the formation of external hydrogen bonds with water is greatly reduced by the presence of the hydrophobic alkyl group and would be distrupted at relatively low surface pressures as was observed (Table II) (see Figure 6E).

With all the phospholipids studied, the presence of a high ionic subphase and the associated Gouy–Stern–Chapman layer restored lateral proton conduction presumably by preventing the formation of internal hydrogen bonds by binding of the cations to the phosphate groups while at the same time maintaining intact the proton conducting hydration layer. Such cation binding to the polar region is consistent with the observed expansion of the monolayer on a 4 M NaCl subphase (Figure 2). Similar expansion of monolayers of PGP and dPGP on a water subphase in the pH range 0–2 and a NaCl concentration from 0.01 to 1 M has been observed (Quinn et al., 1988). In contrast, a condensation of liposome bilayers of PGP formed in the presence of 4 M salt has been reported (Quinn et al., 1986). Presumably, this condensation is linked to the interdigitation of the alkyl chains which occurs only in bilayers.

The results of these experiments show clearly that the central free hydroxyl group of the glycerol moiety in the major phospholipid molecule (PGP) of extreme halophiles plays a critical role in lateral proton conduction, since its deletion from the molecule can result in the termination of proton conduction at high film pressures (Table II; Figure 6).

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Registry No. PGP, 2679-48-3; PG, 42274-15-7; dPGP, 120287-12-9; dPG, 120287-14-1; ddPG, 120287-15-2; STGD, 65941-02-8; H^+, 12408-02-5.

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Transferred Nuclear Overhauser Effect Analyses of Membrane-Bound Enkephalin Analogues by ^{1}H Nuclear Magnetic Resonance: Correlation between Activities and Membrane-Bound Conformations†

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ABSTRACT: Leu-enkephalin, [D-Ala^2]Leu-enkephalin, and [D-Ala^2]Leu-enkephalinamide (agonists) and [L-Ala^2]Leu-enkephalin (inactive analogue) bind to lipid bilayer consisting of phosphatidylcholine and phosphatidylserine. The conformations that these compounds assume, once bound to perdeuterated phospholipid bilayer, have been shown to be unique, as shown by the transferred nuclear Overhauser effect (TRNOE) of ^{1}H NMR spectroscopy. In addition, their location in the bilayer was analyzed by TRNOE in the presence of spin-labeled phospholipids. These analyses showed a clear relationship between the activity and the peptide–membrane interaction. The three active peptides, when bound to membranes, adopt the same conformation, characterized by a type II′ β-turn around Gly^3-Phe^4 and a γ-turn around Gly^2 (or D-Ala^2). The inactive analogue, [L-Ala^2]Leu-enkephalin, displayed a completely different TRNOE pattern corresponding to a different conformation in the membrane-bound state. The tyrosine residue of the active compounds is not inserted into the interior of membrane, but it is inserted into the bilayer for the L-Ala^2 analogue. According to these results, [L-Ala^2]Leu-enkephalin may be explained to be inactive because the mode of binding to the membranes is different from that of active compounds.

Enkephalins are peptides with morphine-like activity that have a primary structure Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin) or Tyr-Gly-Gly-Phe-Met (Met-enkephalin) (Hughes et al., 1975). Many studies have been performed to determine their conformation–activity relationships. At first, enkephalins were assumed to take a conformation analogous to that of morphine, which has a rigid structure, because they bind to the morphine receptor (μ receptor) (Horn & Rodgers, 1976). At present, though biochemical characteristics of enkephalin receptor(s) have not yet been clarified, at least three subtypes of receptors (μ, δ, and κ) have been reported (Paterson et al., 1983). Leu-enkephalin binds to the δ receptor with the highest affinity of those three subtypes, but it also binds to the μ receptor with high affinity (Paterson et al., 1983; Kosterlitz & Paterson, 1985). These receptors have been postulated to couple with one of the inhibitory GTP-binding regulatory proteins (G_i proteins)[1] and to lower the concentration of cyclic

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