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A ^{31}P - NMR STUDY ON MULTILAMELLAR LIPOSOMES FORMED FROM THE LIPIDS OF A THERMOPHILIC BACTERIUM

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SUMMARY: The membrane lipids of a thermophilic bacterium,*Thermus* SPS11, isolated from thermal springs in São Pedro do Sul, Portugal, were fractionated by chromatography on silica gel. The total lipid extract was found to contain one major phospholipid (PL), which accounts for about 90% of the total lipid phosphorous, and one major glycolipid (GL), which accounts for about 95% of the total carbohydrate in the non-phospholipid fraction. The membranes also contain about 11% by weight of a complex mixture of carotenoids (CA). Multilamellar liposomes, in excess water, formed from PL and mixtures of PL with GL and CA in proportions found in the natural membrane were investigated by proton-decoupled ^{31}P-nuclear magnetic resonance (NMR) spectroscopy and X-ray diffraction. All mixtures examined were found to be in a lamellar phase with disordered hydrophobic chains with no evidence for "non-bilayer structures" between 23° and 85°C. Compared to bilayers formed from pure PL or mixtures of PL and CA, significantly larger values for the chemical shift anisotropy of the ^{31}P-NMR powder patterns were obtained from bilayers formed from mixtures of PL and GL, at temperatures above 75°C, and mixtures of PL, GL and CA at all temperatures examined. These differences are interpreted in terms of changes in the order of the bilayer and/or changes in the orientation of the phosphate moiety of PL. The significance of these results to the thermophily of the bacterium is discussed.

The thermophilic bacterium, *Thermus aquaticus*, is a non-sporulating extreme thermophile [1] which grows between about 40° and 80°C with an optimal growth temperature at around 70°C [1-3]. Members of the species have been isolated from the thermal springs of São Pedro do Sul, Portugal [4,5]. Their growth characteristics and the composition of their membrane lipids has been studied in cultures grown between 50° and 78°C [4,5]. Two major polar lipids are encountered, a phospholipid (PL) and a non phosphorous-containing glycolipid (GL). PL accounts for about 90% of the phosphorous and GL accounts for about 95% of the carbohydrate in total lipid extracts of the bacteria. When the bacteria are grown at their optimal growth temperature, between 70° and 75°C, PL and GL are extracted in a molar ratio of about 1:1 assuming that PL has one phosphorous atom and GL has four monosaccharide residues per molecule. In addition, a complex mixture of carotenoids (CA) makes up between 11% and 27% by weight of the total membrane lipid depending upon the strain. In the strain SPS11 the CA content of the membrane is about 11% by weight [4].

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The glycolipid content of a number of *Thermus* species has been shown to increase with increasing growth temperature [6,7]. In the strain SPS11 an increase in the GL/PL ratio has also been observed when the bacteria are grown at 78°C as compared to those grown at 50°C [4,5]. This observed increase in the proportion of glycolipid in the membrane taken along with the observation that glycolipids "rigidify" phospholipid bilayers [8,9], has led to the interesting speculation that they "may play a major role in stabilizing membranes of thermophiles to thermal and other environmental stresses" [3]. In the present study we have attempted to lend substance to this speculation by studying the physical properties of bilayers (as multilamellar liposomes) prepared from lipids of *Thermus* SPS11 by ^{31}P-nuclear magnetic resonance (NMR) spectroscopy and X-ray diffraction. Our results support the speculation and indicate a possibly crucial role for the carotenoids as well.

MATERIALS AND METHODS

Thermus SPS11 was grown aerobically at 72°C to late exponential phase in a 25 liter fermentor (New Brunswick Scientific Co., model MF-128S) using the growth medium described by Brock [2].

Lipids were extracted using the procedure of Bligh and Dyer [10]. The chloroform extract was washed with water to remove non-lipid impurities. The washed total lipid extract, dissolved in a minimal volume of chloroform/methanol (1:1, by vol.) was applied onto a column of silica gel (Kieselgel 60, particle size 0.063-0.200 mm, E. Merck, Darmstadt, F.R.G.) equilibrated and subsequently eluted with chloroform/methanol (95:5, by vol.). The CA were eluted at the solvent front. The polar lipid fraction adsorbed on the column was eluted as one band by washing with chloroform/methanol/water (65:25:4, by vol.). The solution was dried by addition of anhydrous sodium sulfate and the solvent was removed by rotary evaporation. The residue, dissolved in a minimum volume of chloroform/methanol/25% ammonia/water (70:30:4:1, by vol.) was applied onto a column of silica gel equilibrated and eluted with the same solvent. The major phospholipid, PL, was the first band eluted. Elution was continued until the minor phospholipids (2nd. band) and minor glycolipids (3rd. band) were eluted and the column was then developed with chloroform/methanol/water (65:25:4, by vol.) until the major glycolipid, GL, band was eluted. Each of the bands were collected separately, and evaporated to dryness by rotary evaporation. The residues were dissolved in chloroform/methanol (1:1, by vol.) and stored at -18°C.

Phospholipid was estimated by the method of Fiske and Subbarow [11]. Glycolipids were estimated by the phenol-sulfuric acid method [12]. GL has been shown to have four monosaccharide (3 glucose and 1 glucosamine) residues per molecule [4]. CA were estimated gravimetrically. In preparation of liposomes, the desired amount of lipid or lipid mixture was evaporated to dryness by rotary evaporation and then stored in a vacuum dessicator overnight to remove all traces of the solvent. The dry residue was hydrated by gently shaking with 5 mL of 5 mM Tris-HCl buffer, pH7.9 / 150 mM NaCl. the suspension was centrifuged for 10 min at 10,000 x g and only the pellet was used in the NMR or X-ray diffraction experiments.

Proton-decoupled ^{31}P-NMR spectra were obtained at 81 MHz on a Varian XL-200 FT-NMR spectrometer using a single pulse experiment. Between 5,000 and 7,000 transients were accumulated for each spectrum. ^{31}P chemical shifts, in ppm, were referenced to external H_3PO_4. Thin layer chromatographic analysis of the samples, after obtaining the NMR spectra, did not show any signs of lipid decomposition. X-ray diffraction analysis was performed as described by Stümpel *et al* [13].

RESULTS AND DISCUSSION

Pellets obtained by centrifugation of aqueous suspensions of PL; a 1:1 molar ratio mixture of PL and GL; PL containing 11% by weight of CA; and a 1:1 molar mixture of PL and GL containing 11% by weight of CA; the last mixture reflecting the composition of the natural membrane, were subjected to X-ray diffraction analysis at temperatures between 5° and 75°C. In all cases, above 25°C the reflexes in the low and wide angle region were typical of a lamellar phase with disordered apolar chains (Lα phase). The characteristic spacings at 50°C for all the systems studied are summarized in Table I.

Proton-decoupled ^{31}P-NMR spectra for the same set of samples were obtained between 23° and 85°C. Figure 1 shows typical spectra obtained from the 1:1 molar ratio mixture of PL and GL containing 11% by weight of CA over this temperature range. The powder patterns are characteristic of lipid-water systems in the Lα phase [14-16]. Interestingly, no evidence for the existence of so-called "non-bilayer lipid" structures [16] was seen even at the highest temperatures examined. This was the case in all mixtures examined with the possible exception of PL containing 11% by weight of CA where a hint of an isotropic component was seen (see Figure 2).

The chemical shift anisotropy, $\Delta\nu_{CSA}$, of the ^{31}P-NMR spectra as a function of temperature is shown in Figure 3 for the four cases examined. The value of $\Delta\nu_{CSA}$ obtained for dispersions of PL alone is about 35 ppm at 25°C decreasing monotonically to about 30 ppm at 85°C. The mixture of PL with 11% by weight of CA shows a similar result. The values of $\Delta\nu_{CSA}$ in this case are slightly lower than those for pure PL dispersions upto about 55°C but

Table I

X-ray diffraction data from multilamellar liposomes of Thermus SPS11 lipids at 50° C

Lipid	d(Å)	s(Å)
PL	76,3	4,63
PL + CA	77,7	4,67
PL + GL	69,0	4,66
PL + GL + CA	59,8	4,61
GL	65,0	4,70

Errors in measurement of d are ± 0,5 Å and of s are ± 0.05Å. PL = phospholipid alone; GL = glycolipid alone; PL + CA = phospholipid with 11% (by wt.) carotenoid; PL + GL = a 1:1 (molar) mixture of phospholipid and glycolipid; PL + GL + CA = a 1:1 (molar) mixture of phospholipid and glycolipid containing 11% (by wt.) carotenoid.

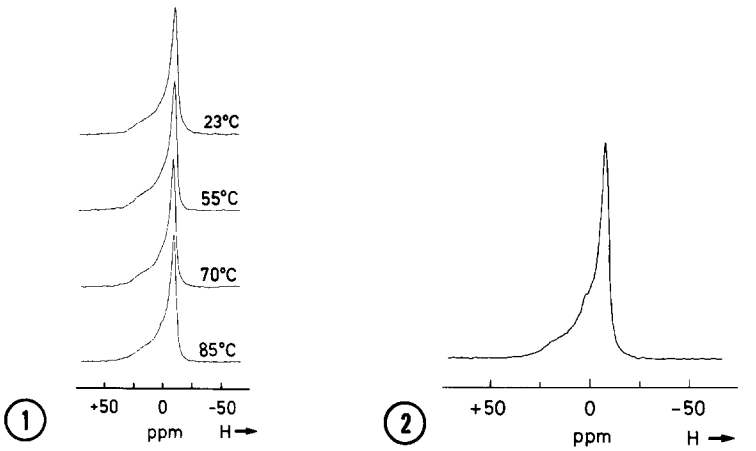

Figure 1: Proton-decoupled ^{31}P-NMR spectra at 81 MHz of an aqueous suspension (liposomes) of a 1:1 molar ratio mixture of PL and GL containing 11% by weight of CA. The suspensions were prepared in 0.15 M sodium chloride / 5 mM Tris-hydrochloride buffer, pH 7,9 / 20% deuterium oxide. The temperatures are indicated for each spectrum in the figure.

Figure 2: Proton-decoupled ^{31}P-NMR spectrum of an aqueous suspension (liposomes) of PL containing 11% by weight of CA, at 85°C. The suspension was prepared in 0.15 M sodium chloride / 5 mM Tris-hydrochloride, pH 7.9 / 20% deuterium oxide.

are higher at temperatures above 75°C. The mixture of PL and GL in a 1:1 molar ratio shows a $\Delta\nu_{CSA}$ value of about 37 ppm at 25° and 55°C, and a large increase between 55° and 75°C to a value of about 43 ppm above the latter temperature. It may be of interest to recall here that the signal comes from the PL alone since GL does not have phosphorous. The mixture of PL and GL at a 1:1 molar ratio with 11% by weight of CA shows a constant $\Delta\nu_{CSA}$ value of about 40 ppm at all temperatures examined in this work.

The largest values of $\Delta\nu_{CSA}$ obtained in the present study are for a 1:1 molar ratio mixture of PL and GL, at temperatures above 75°C, and for the same mixture with the addition of 11% by weight of CA, at all temperatures examined. These values are comparable

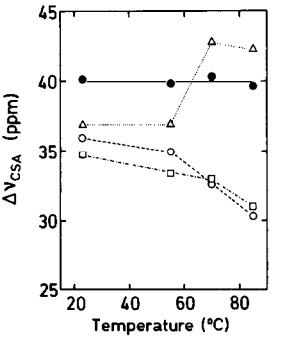

Figure 3: Temperature dependence of the chemical shift anisotropy ($\Delta\nu_{CSA}$) of the various liposome preparations of *Thermus* SPS11 lipids. (○) PL; (□) PL containing 11% by weight of CA; (△) a 1:1 molar ratio mixture of PL and GL; (●) a 1:1 molar ratio mixture of PL and GL containing 11% by weight of CA.

to those obtained with aqueous dispersions of synthetic phosphatidylcholines in their Lα phase in excess water [14]. On the other hand, we observe particularly low values for dispersions of PL and PL with 11% CA by weight. Changes in ΔνCSA may, in principle, arise from a change in the orientation of the director axis relative to the bilayer plane and/or a change in its motional amplitude. The two possibilities can only be distinguished by studying oriented samples. If the low values are understood in terms of an increased motional amplitude of the phosphate moiety about its director axis, this is equivalent to an increased disorder in the bilayers formed from pure PL and PL with 11% CA. If this is the case, it is clear that the glycolipid has the effect of reducing the motional amplitude of the wobbling motion or increasing the order in the bilayer. Since GL and PL have similar apolar portions [4], the cause must be looked for in the polar part of the molecule. One possibility is that the hydrogen bonding between the glycosidic residues among themselves and with water increases the order at the bilayer-water interface. This proposed ordering of the interface would have the effect of reducing the cone angle of wobbling motions of all lipids in the bilayer as long as there is no phase separation in the system.

In this context, the particular case of dispersions of the PL + GL mixture which show low ΔνCSA values below 55°C and high values above 75°C suggest that there may be a phase separation of PL and GL within the bilayers below 55° so that the PL-rich domains behave similarly to pure PL bilayers. Above 75° the PL and GL domains mix and the ordering effect of GL is felt by the PL molecules. The change in ΔνCSA observed between 55° and 75°C could, of course, also be due to a reorientation of the PL phosphate group induced by GL. Even if this were the case, a phase separation below 55°C is suggested. The high values of ΔνCSA at all temperatures examined in the mixture of PL, GL and CA suggests that no phase separation of PL and GL occur in this mixture, presumably an effect of the CA.

In conclusion we speculate on the relevance of the results reported here to the thermophily of *Thermus* SPS11. The high glycolipid content of the membrane suggests a structural role for this lipid. The results reported here and from other laboratories [3,8,9] indicate that the glycolipid serves to stabilize the membrane at high temperatures. Our results also indicate that this stabilizing role is only evident above 75°C where, as has been argued above, a mixing of glycolipid and phospholipid take place. Below 55°C these lipids seem to be phase separated and no stabilization of the phospholipid domains is evident. Further, it may be expected that a massive phase separation in the membrane would be lethal to the organism both from a point of view of membrane-protein function and from a point of view of membrane permeability at the domain-domain interfaces. The results also indicate that CA in the membrane serves to avoid phase separation of the glycolipid, thereby extending its stabilizing effect to temperatures as low as 23°C. This could be particularly important for the survival of the organism if its environmental temperature should fall below 55°C.

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