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A spin label ESR and saturation transfer-ESR study of archaebacteria bipolar lipids

S. Bruno[1], S. Cannistraro[2], A. Gliozzi[1]*, M. De Rosa[3], and A. Gambacorta[3]

[1] Dipartimento di Fisica, I-16146 Genova, Italy,
[2] Dipartimento di Fisica, I-06100 Perugia, Italy, and
[3] Istituto per la Chimica di Molecole di Interesse Biologico, I-80072 Arco Felice (Na), Italy

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Abstract. A spin label study has been carried out on bipolar lipids extracted from *Sulfolobus solfataricus*, an extreme thermophilic archaebacterium growing at about 85 °C and pH 3. These lipids are cyclic diisopranyl tetraether molecules, quite different from the usual fatty acid lipids. Two hydrolytic fractions of the membrane complex lipids have been studied: the symmetric lipid glycerol-dialkyl-glycerol-tetraether (GDGT) and the asymmetric lipid glycerol-dialkyl-nonitol-tetraether (GDNT). The ESR spectra confirm the results previously obtained from calorimetric and X-ray diffraction experiments showing a polymorphic behaviour of these lipids and indicating the critical temperature ranges at which structural transitions occur. Moreover, the present study adds information on the dynamics of the different portions of the hydrophobic chain. ST-ESR measurements show correlation times ranging from 10^{-8} s up to 10^{-5} s, depending upon the lipid sample, the label position and the degree of hydration. At very high temperatures, i.e. the physiological temperatures of *Sulfolobus solfataricus*, the nonitol head groups of the asymmetric lipids form a strongly immobilized structure. Indeed, the molecular correlation times of the outermost hydrophobic portion of GDNT are higher, by a factor up to 10^3, than those of usual monopolar lipids. Anisotropic motional behaviour is observed even at such very high temperatures. Possible biological implications are discussed.

Key words: Archaebacteria, bipolar lipids, ESR, ST-ESR, spin labeling

Introduction

Archaebacteria are very interesting microorganisms from the point of view of the evolution of life. Indeed, they live under those extreme conditions that seem characteristic of the early history of life: methanogens in the absence of oxygen, halophiles at high salt concentration, thermophiles at very high temperature. Recent phylogenetic analyses have led some authors to propose archaebacteria as a new primary kingdom. Further support for such argument has been given by the observation of the presence, in most ancient petroleums, of branched hydrocarbon chains of the phytanil type, which are similar to those exhibited by archaebacteria plasma membrane lipids (Chappe et al. 1982). We studied the lipids of *Sulfolobus solfataricus*, an extreme thermoacidophilic archaebacterium, the natural habitat of which is at 90 °C and pH 2. A pH difference exists across the membrane of up to 4 pH units. The chemical structure of its lipids is remarkably different from that of the usual ones. They consist of two C_{40} $\omega-\omega'$ biphytanyl residues having two polar heads (two glycerol or one glycerol and one nonitol), as sketched in Fig. 1. The free hydroxyl group of glycerol and one of nonitol may be substituted by a variety of polar groups (De Rosa et al. 1986). The hydrophobic chain of these lipids may contain from zero up to four cyclopentane rings per chain (De Rosa et al. 1983a). Previous work, both in vivo (De Rosa et al. 1983b) and in black membranes (Gliozzi et al. 1983a), has shown that these lipids are arranged in monolayers lacking a midplane region. Structural rearrangements deduced from changes in transport properties and in electrical capacitance have been observed in these monolayer black membranes (Gliozzi et al. 1983a). Moreover, structural aspects and phase

* To whom offprint requests should be addressed

Abbreviations used are: ESR, electron spin resonance; St-ESR, saturation transfer electron spin resonance; GDGT, glycerol-dialkyl-glycerol-tetraether; GDNT, glycerol-dialkyl-nonitol-tetraether; 5SASL, 12SASL and 16SASL, stearic acid spin labels, N-oxyl-4′,4′-dimethyloxazolidine derivatives of 5-ketostearic acid, 12-ketostearic acid and 16-ketostearic acid, respectively; DSC, differential scanning calorimetry

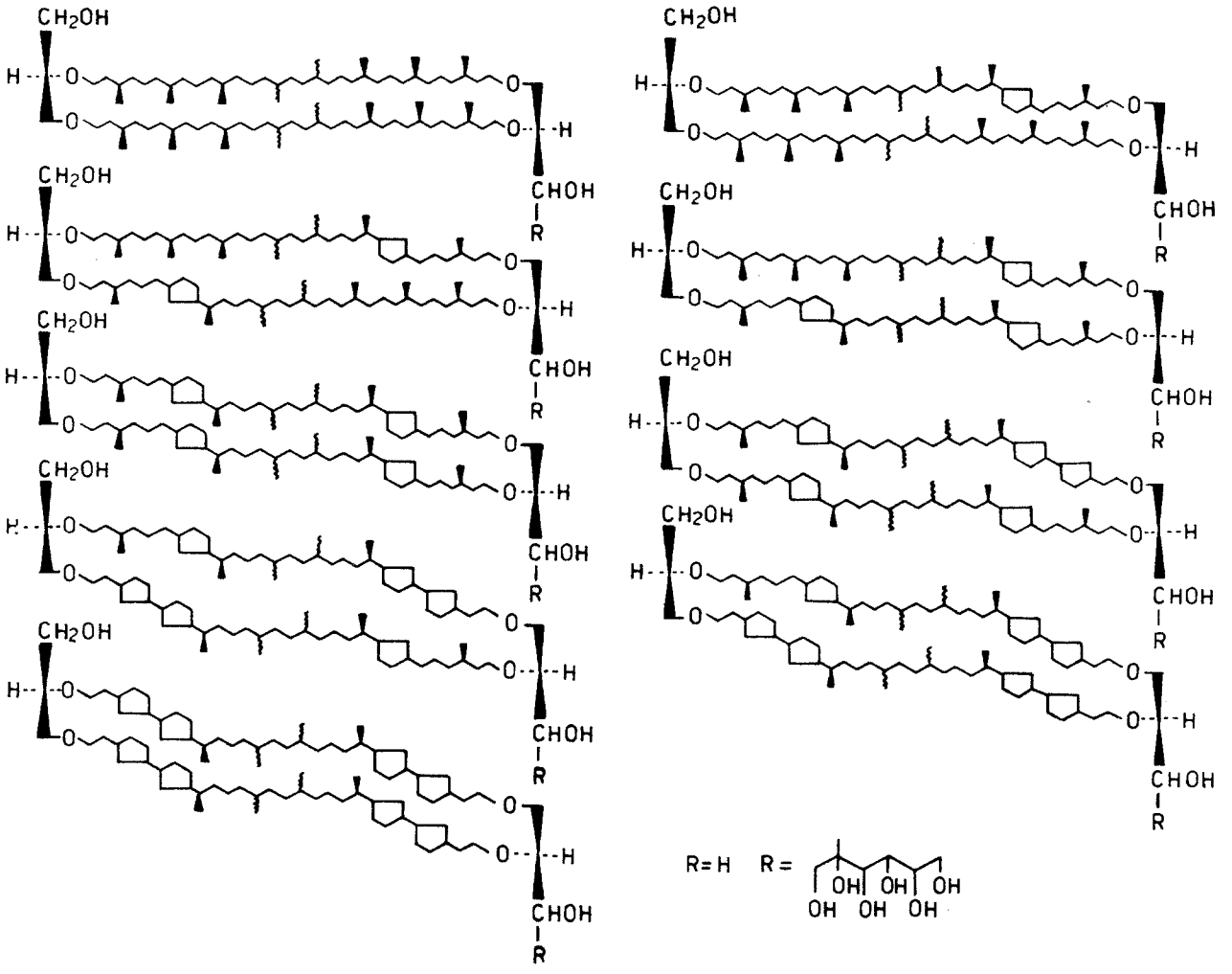
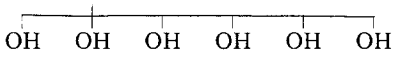


Fig. 1. Structure of bipolar isopranyl ethers, the backbone of the complex lipids of *Sulfolobus solfataricus*. R=H in glycerol-dialkyl-glycerol-tetraether (GDGT)

R = 

in glycerol-dialkyl-nonitol-tetraether (GDNT). Black arrows and wriggles mean respectively known and unknown chirality (from De Rosa et al. 1983a)

transitions related to polymorphism of these lipids have been elucidated by calorimetric studies (Gliozzi et al. 1983b) and X-ray diffraction techniques (Gulik et al. 1985). The present Electron Spin Resonance (ESR) and Saturation Transfer-ESR (ST-ESR) study extends this knowledge, casting a new light on the dynamic aspect at a molecular level. ESR and ST-ESR spectra of extrinsic spin probes, e.g. spin-labelled fatty acids, introduced in bipolar lipid samples have been analyzed as a function of temperature, of hydration and of free radical position along the chain.

Theoretical considerations

The mobility of a lipid system can be represented in terms of the rotational correlation time, τ_c. If τ_c is lower than 10^{-8} s, dynamic information on the lipid system can be obtained from the order parameter, S. This parameter can be expressed by the relationship (Hubbell and McConnell 1971)

$$S = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - 1/2\,(A_{xx} + A_{yy})}\, k\,, \tag{1}$$

where

$$k = \frac{A_{\parallel} + 2A_{\perp}}{A_{xx} + A_{yy} + A_{zz}} \tag{2}$$

is a correction for polarity changes (Griffith and Jost 1976). The values of $A_{\parallel}$ and $A_{\perp}$ are obtained by

$$A_{\parallel} = A_{\max}$$

$$A_{\perp} = A_{\min} + 1.32 + 1.86 \log (1 - S_{\text{app}})\,,$$

where A_{max} and A_{min} are the outer- and the inner-hyperfine splitting respectively (shown in Fig. 2), A_{xx}, A_{yy}, A_{zz} are the principal hyperfine coupling constants of 6.3, 5.8 and 33.6 Gauss respectively (Griffith and Jost 1976) and

$$S_{app} = \frac{A_{max} - A_{min}}{A_{zz} - 1/2\,(A_{xx} + A_{yy})}. \quad (3)$$

If τ_c is higher than 10^{-8} s, the order parameter is overestimated, and the spectral splitting parameter $2A_{\parallel}$ may be used to obtain semi-quantitative information on the dynamic behaviour of the biosystem. This procedure, however, is correct up to $\tau_c \lesssim 10^{-7} - 10^{-6}$ s, i.e. in the so called slow motional region, since above this higher limit for τ_c conventional ESR can no more resolve changes in τ_c. ST-ESR is therefore needed for systems undergoing molecular motions in the very slow time domain ($\tau_c \gtrsim 3 \cdot 10^{-7}$ s). Moreover, this non-linear ESR technique is sensitive for τ_c values down to 10^{-8} s.

The best procedure to obtain reliable values of τ_c from experimental ST-ESR spectra, both for isotropic and anisotropic systems, has been proven to be the computer simulation. But current simulation of ST-ESR spectra of nitroxide spin labels requires a great deal of computer time and memory (Perkins et al. 1976; Robinson and Dalton 1980). An alternative method to obtain rotational correlation times has been developed by Thomas et al. (1976). They considered that ST-ESR spectra can be well characterized by three independent lineshape parameters, L''/L, C'/C and H''/H. These spectral parameters have been calibrated against τ_c by applying the Debye equation for Brownian rotational diffusion to spin-labelled macromolecules. Therefore, τ_c values can be obtained empirically from these calibration curves. Although this method is correct only for isotropic molecular motion, analysis of anisotropic motion has been widely performed by using the isotropic data of reference (Hyde and Dalton 1979; Marsh 1980; Marsh and Watts 1980). The latter procedure leads to a discrepancy between τ_c values arising from different spectral ratios. A possible interpretation is that $\tau_c(C'/C)$ yields information on the motion around the long molecular axis of the spin label, whereas $\tau_c(L''/L)$ and $\tau_c(H'/H)$ are the correlation times for the motion of the long axis itself (Marsh 1980).

Materials and methods

Glycerol-dialkyl-glycerol-tetraether (GDGT or symmetric lipid) and glycerol-dialkyl-nonitol-tetraether (GDNT or asymmetric lipid) were extracted from *Sulfolobus solfataricus* and purified according to the procedures previously described (De Rosa et al. 1983 a). The composition of the lipid native mixture was determined and the results are listed below. The values in the brackets represent the number of pentanic rings per each chain. Symmetric lipid: (2+2) 23.2%; (3+3) 36%; (3+2) 40.7%; (3+4) traces. Asymmetric lipid: (2+2) 26.6%; (3+3) 32.6%; (3+2) 44.8%; (3+4) traces. In some experiments we used a separated fraction of GDGT containing a very low amount of cyclopentane rings (about one ring per molecule). Egg lecithin lipids were purchased from Sigma Chem. Co., St. Louis and 4,4-dimethyloxazolidinyl nitroxides derived from 5-keto, 12-keto and 16-keto stearic acids (5SASL, 12SASL and 16SASL, respectively) were purchased from SYVA Res. Chem., Palo Alto. Stock solutions of GDNT, GDGT and egg lecithin were prepared by dissolving the lipids in chloroform at a concentration of 10 mg/ml while the spin labels were dissolved in ethanol. To prepare ESR samples, aliquots of label solution were added to the lipid solution to reach different label/lipid molar ratios. Optimal label/lipid molar ratio in order to minimize dipolar interactions and maximize signal amplitude was found to be about 1%. With both lipids we have not succeeded in obtaining oriented samples, neither by using standard methods, nor by means of electric or magnetic fields, up to $3.5 \cdot 10^3$ V/cm and $6 \cdot 10^3$ G, respectively.

For the conventional ESR study, samples were prepared by introducing 50 µl of the lipid-label mixture at the bottom of a standard ESR quartz tube, 5-cm long, and by evaporating the solvent under vacuum overnight, thus obtaining the dry samples. Hydrated samples were prepared by adding a few drops of water to the dry samples and removing the excess water after at least 30 min. In both cases sample tubes were sealed. We were particularly careful, in preparing the samples for ST-ESR measurements, to minimize their sizes at the bottom of the ESR quartz tube. In fact ST-ESR spectra strongly depend upon the sample size and position due to inhomogeneities in the microwave and modulation field in the resonant cavity (Fajer and Marsh 1982; Delmelle 1983). No macroscopic condensation was observed on the internal tube walls when the hydrated sample was examined at high temperatures during a temperature run.

The standard ESR quartz tubes were put in the temperature control quartz dewar so as to locate the sample exactly in the centre of the resonant TE_{102} cavity of the spectrometer. The sample temperature was regulated by a nitrogen flux and monitored by a platinum thermo-resistor placed immediately below the ESR tube. Temperature gradient across the sample was found not to exceed 0.5 °C/cm. Mea-

surements were performed at thermodynamic equilibrium, i.e. no hysteresis effects, depending on direction of temperature variation were detected. The latter state was reached by equilibrating the sample for 6 min after changing temperature.

ESR spectra were recorded by an X-band Varian E-109 spectrometer. A 100 kHz modulation frequency was used for conventional first harmonic, in-phase, absorption ESR spectra. ST-ESR spectra were recorded in the second harmonic, 90° out-of-phase, absorption mode at a modulation frequency of 50 kHz, receiver frequency of 100 kHz, modulation amplitude of 5 Gauss and microwave magnetic field strength of 0.25 Gauss. The detailed calibration of these two latter experimental settings, upon which ST-ESR lineshapes are critically dependent (Beth et al. 1979; Fajer and Marsh 1982; Delmelle 1983), has been performed as will be described elsewhere (Bruno et al. in preparation). The microwave power in the waveguide was measured with a Marconi 6960 powermeter in conjunction with the 6910 head. The phase was set by using the "self-null" method (Thomas et al. 1976) at 1 mW power level. To calculate the experimentally observed g-factors, a magnetic field calibration was performed with a Magnion Precision NMR Gaussmeter, the microwave frequency being measured with a Marconi 2440 counter.

Data presented below are based on repeated experiments on different samples, which proved to be quite reproducible. Therefore to make the trends clearer results are not mediated on different measurements, but are given on a single experiment.

Results

Conventional first harmonic, in-phase, absorption ESR spectra, recorded at different temperature values, of dry GDGT and GDNT samples labelled both with 5 and 16SASL, are shown in Fig. 2. As can be immediately seen, the 5SASL-labelled GDNT spectra exhibit rigid-limit powder shapes, at variance with the GDGT ones.

A ST-ESR check performed at room temperature on GDGT samples, both dry and hydrated, showed that, at this temperature, correlation time is of the order of 10^{-8} s for the 5SASL and even lower for the 16SASL spin label. Conventional ESR then allows an estimate of the order parameter, S. Figure 3 shows the temperature-dependence of S for dry and hydrated samples of symmetric lipids labelled with 5, 12 and 16SASL, respectively. At variance with normal lipids the order parameter does not change in a significant way by increasing the deepness along the hydrophobic chain up to position C_{12},

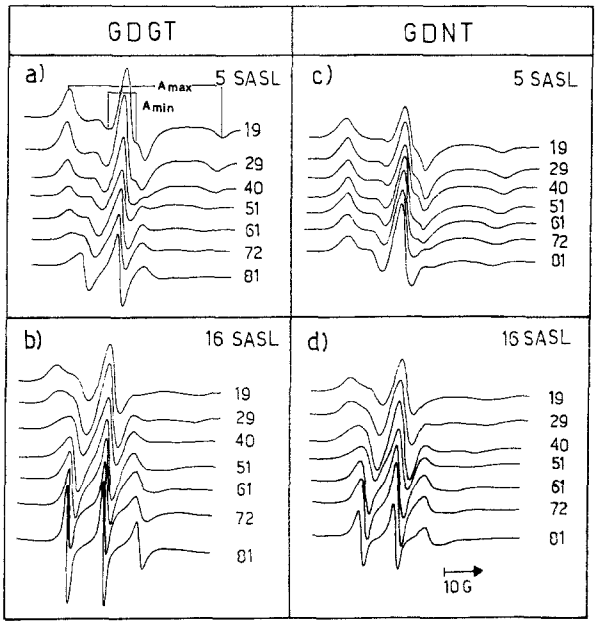


Fig. 2a–d. Conventional first harmonic, in-phase, absorption ESR spectra recorded at different temperatures values of **a** dry GDGT labelled with 5SASL; **b** the same as **a** but labelled with 16SASL; **c** dry GDNT labelled with 5SASL; **d** the same as **c** but labelled with 16SASL. The samples are randomly oriented and label/lipid molar ratio is about 1%. ESR settings as follows: magnetic field sweep rate 100 Gauss in 4 min, time constant 0.25 s, modulation amplitude 1.25 Gauss and microwave power level 8 mW

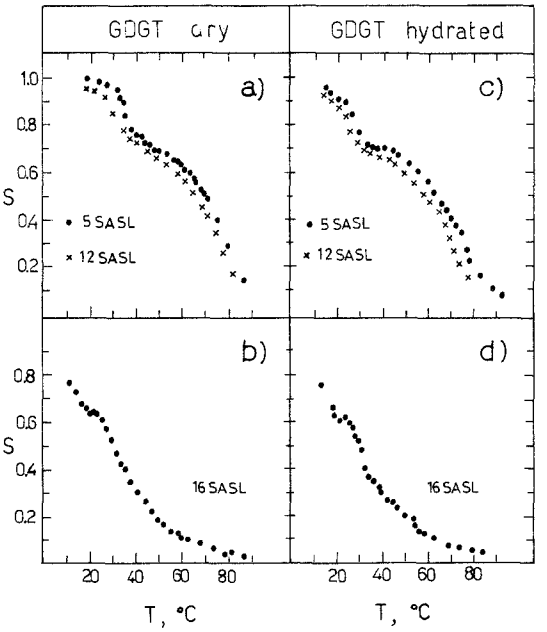


Fig. 3a–d. Temperature dependence of the order parameter S of **a** dry GDGT labelled with 5SASL and with 12SASL (*circles* and *crosses*, respectively); **b** the same as **a** but labelled with 16SASL; **c** hydrated GDGT labelled with 5SASL and with 12SASL (*circles* and *crosses*, respectively); **d** the same as **c** but labelled with 16SASL. Sample conditions and ESR settings as in Fig. 2. Experimental errors do not exceed circle diameter and crosses size

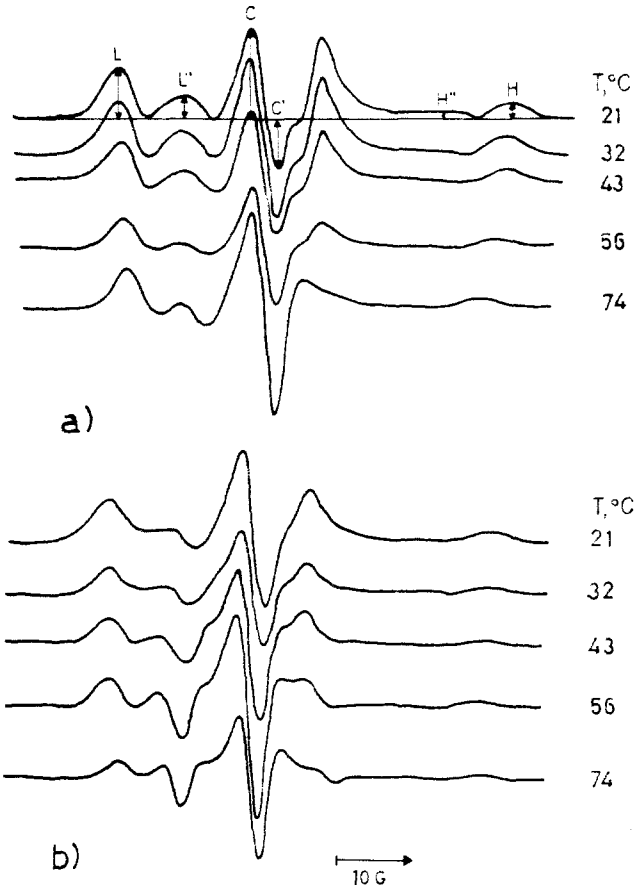


Fig. 4a and b. ST-ESR spectra recorded at different temperature values in the second harmonic, 90° out-of-phase, absorption mode of dry **a** and hydrated **b** GDNT samples labelled with 5SASL. The samples are randomly oriented and label/lipid molar ratio is about 1%. ESR settings as follows: magnetic field sweep rate 100 Gauss in 4 min, time constant 0.25 s, modulation amplitudes 5 Gauss and microwave field strength 0.25 Gauss

whereas significant changes both in the temperature trend and in S values are obtained at C_{16}. The hydrophobic part of these lipids differs from the usual one both for the presence of cyclopentane rings up to position C_{12} and of methyl groups. To test the relative importance of these two factors, measurements were repeated using a lipid fraction containing a very low amount of rings (a mean value of $\cong 1$ ring per molecule, against $\cong 5$ in the native mixture). Even in this case the order parameter of the 5SASL- and the 12SASL-labelled GDGT was quite similar, whilst the 16SASL showed much lower values. This fact indicates the important role played also by the methyl groups in conferring rigidity to the outermost portion of the hydrophobic chain. As expected, however, the order parameter was lower than the corresponding value of the native mixture. For instance at 20 °C it was 0.97, 0.95 and 0.52 for 5, 12 and 16SASL, respectively, against 0.995, 0.95 and 0.65 of the native mixture.

Under dry conditions, we identify a discontinuity in the S curve of the 16SASL-labelled sample at about 24 °C and a sudden drop centred at about 35 °C for the 5SASL-labelled one. The hydrated sample behaves similarly, but these two critical points are slightly shifted: hydrated 16SASL-labelled sample shows the transition temperature at 26 °C and the 5SASL one at 30 °C. Furthermore, at $T \gtrsim 60$ °C the decrease rate of S values becomes higher for 5SASL than for 16SASL, in both dry and hydrated samples.

At variance with the GDGT case, the 5SASL-labelled GDNT, both dry and hydrated, exhibit a very high immobilization. Figure 4 shows the second harmonic, 90° out-of-phase, absorption ESR spec-

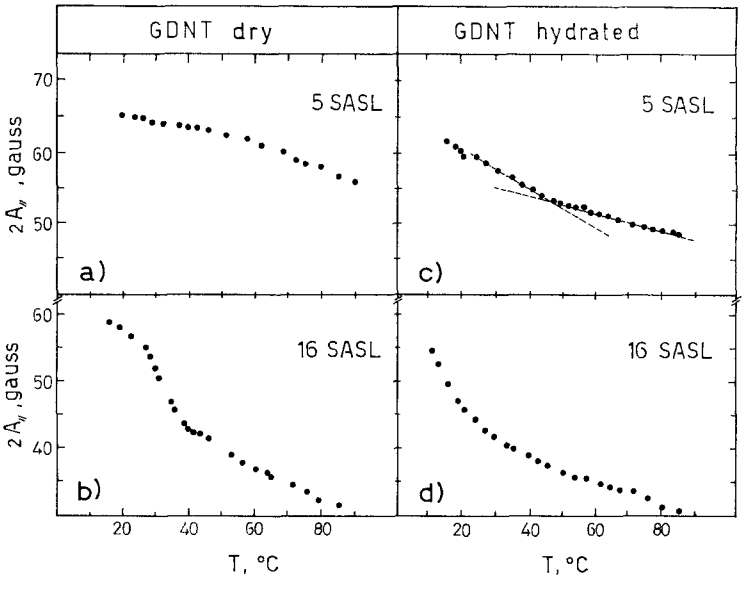


Fig. 5a–d. Temperature dependence of the maximum hyperfine splitting $2A_{\parallel}$ of **a** dry GDNT labelled with 5SASL; **b** the same as **a** but labelled with 16SASL; **c** hydrated GDNT labelled with 5 SASL; **d** the same as **c** but labelled with 16SASL. Sample conditions and ESR settings as in Fig. 2. Experimental errors as in Fig. 4

Table 1. Experimental hyperfine splittings from different lipid samples for the temperature range 20° – 85 °C

Temperature [°C]	$2A_{\parallel}$ [Gauss]									
	Egg lecithin + 5SASL		GDGT + 5SASL		GDNT + 5SASL		GDGT + 16SASL		GDNT + 16SASL	
	dry	hydrated*	dry	hydrated	dry	hydrated	dry	hydrated	dry	hydrated
20	60	51.5	61.5	62.5	**	61.5	56.5	54.5	57	46
31	57	49.5	60	60.5	**	58.5	48	40.5	49	41.5
37	56	49	59	59	**	57	41.5	39	45.5	40
41	55	48.5	58	58.5	**	56	38.5	37	42.5	38.5
53	53	–	55	54	62	53.5	33.5	33.5	39	36
65	49.5	–	50.5	48.5	60	52	32.5	32.5	36	35
75	46	–	45	41.5	59	50.5	31.5	31.5	34	33
85	42.5	–	37.5	34	57.5	49.5	31	31	32	31

* From Gordon and Sauerheber (1977)
** Data not reported because of very slow motional time domain

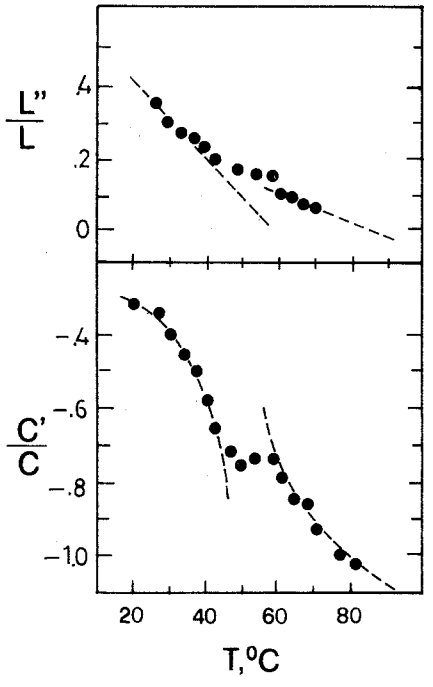


Fig. 6. Temperature dependence of the spectral ratios L''/L (*top*) and C'/C (*bottom*) from ST-ESR spectra of dry 5SASL-labelled GDNT. Sample conditions and ESR settings as in Fig. 5. Experimental error does not exceed circle diameter

tra, which indicate τ_c values ranging in the slow up to the very slow time domain. Consequently, an order parameter study of GDNT would be meaningless. Therefore, we have analyzed the spectral splitting parameter $2A_{\parallel}$. Figure 5 shows the temperature dependence of this maximum hyperfine splitting $2A_{\parallel}$ for dry and hydrated samples of asymmetric bipolar lipids. The values for the 5SASL-labelled dry lipid are overestimated for $T \lesssim 55$ °C, because of the very slow τ_c values. For this reason we will not comment this set of data. The 16SASL-labelled dry system exhibits a slope change in the $2A_{\parallel}$ curve around 40 °C. For hydrated samples one can identify a change in slope with the 5SASL at $T = 50$ °C and a slight irregularity at 22 °C. The 16SASL curve exhibits a rapid decrease up to $T \cong 25$ °C.

The comparative behaviour of the various lipid systems is summarized in Table 1, which collects the $2A_{\parallel}$ values at several temperatures for the dry and hydrated samples of GDGT and GDNT lipids, labelled with 5 and 16SASL. The 5SASL-labelled egg lecithin values are also reported.

To get information about conformational changes also on 5SASL-labelled dry GDNT, we performed a ST-ESR study. The plots of L''/L and C'/C (H''/H is not measurable, as already noted by other authors (Delmelle et al. 1980)) *vs.* temperature are given in Fig. 6. A plateau, particularly clear in the C'/C curve, is exhibited in the range 40–60 °C, suggesting that the system undergoes a broad structural transition at these temperatures.

Table 2 gives the values of C'/C and L''/L, at various temperatures, and the corresponding correlation times. The most peculiar features suggested by these data are the anisotropy of the motion and its remarkably high τ_c values. The presence of anisotropic motion is suggested by the discrepancies between $\tau_c(L''/L)$ and $\tau_c(C'/C)$ and is confirmed by the plot of L''/L *vs.* C'/C, for increasing temperatures (not shown), as indicated by Johnson et al. (1982). The lipid remains in this condition of motional anisotropy at least up to 70 °C (at higher temperatures L'' is no longer measurable).

ST-ESR also indicated anisotropy in the 5SASL-labelled hydrated GDNT. Moreover, in spite of

Table 2. C'/C and L''/L ratios from ST-ESR spectra of 5SASL-labelled GDNT, and the related correlation times, in the temperature range 20° – 80 °C

T[°C]	GDNT + 5SASL dry			
	C'/C	$\tau_C(C'/C)$*	L''/L	τ_c (L''/L)*
20	– 0.28	$3 \cdot 10^{-6}$	0.4	10^{-5}
30	– 0.39	$2 \cdot 10^{-6}$	0.3	$6 \cdot 10^{-6}$
40	– 0.55	10^{-6}	0.25	$3.5 \cdot 10^{-6}$
55	– 0.73	$3 \cdot 10^{-7}$	0.17	$1.5 \cdot 10^{-6}$
70	– 0.9	$1.8 \cdot 10^{-7}$	0.1	$5 \cdot 10^{-7}$
80	– 1	$1.3 \cdot 10^{-7}$	**	**

* Correlation times were calculated through the calibration curves of Thomas et al. (1976)
** Values not measurable on the spectra

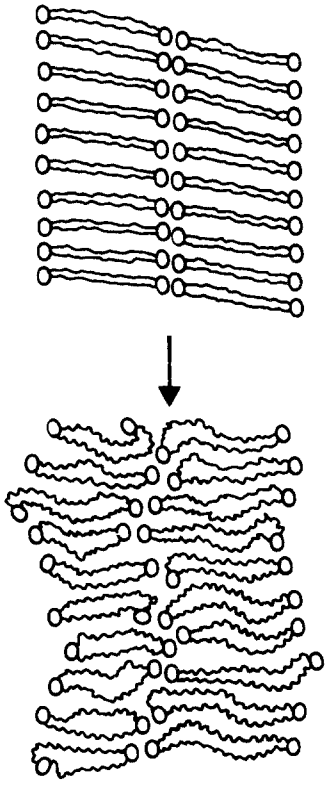


Fig. 7. Schematic drawing of the GDGT phase transition observed around 25 °C

hydration, correlation times preserve high values, i.e. $\tau_c(C'/C) = 3 \cdot 10^{-7}$ s, 10^{-7} s and 10^{-8} s at 20°, 40° and 75 °C, respectively.

Discussion

Before discussing the present set of results, it seems necessary to analyze the question of the spin-labelled stearic acid localization in the bipolar lipid system. It is well known that in normal monopolar lipid systems these amphiphylic probes incorporate by interaction of their hydrocarbon chains with those of the lipid molecules and by interaction of their carboxylic end groups with the lipid polar heads (Sanson et al. 1976). A new question arises concerning the side of insertion in the case of the asymmetric lipid, owing to the double presence of head groups in the bipolar lipids. Remembering that the nonitol head groups tend to cluster in all the lipid phases so far observed (Gulik et al. 1985), we suggest that the spin labels are mostly anchored at the nonitol side. In fact, should the spin-labelled stearic acids face the glycerol head groups, the resonance spectra would be very similar to those obtained with the symmetric lipids. Furthermore, a statistical insertion on both sides would result in a superposition of two different spectra. Neither of these effects is indeed observed.

GDGT

This lipid sample absorbs a very limited amount of water (two water molecules per lipid). Accordingly, data of Table 1 exhibit a very high value of the low temperature hyperfine splitting of the hydrated GDGT sample labelled with 5SASL as compared to that of an egg lecithin dispersion (Gordon and Sauerheber 1977). This indicates the occurrence of a highly ordered structure. The small amount of absorbed water does not greatly affect the lipid organization, very similar behaviour being exhibited by the dry and hydrated samples, with small differences in transition temperatures. In the following we shall discuss in detail only one of them, i.e. the dry one. X-ray diffraction analysis (Gulik et al. 1985) has shown that below 20 °C lipids are arranged in a planar lamellar rigid structure, L_β, in which the chains are stiff and probably not straight. A first order transition occurs at about 24 °C, as indicated by DSC measurements (Gliozzi et al. 1983b). The break observed in the 16SASL-labelled sample at $T = 23$ °C (Fig. 4) corresponds to such a transition. Above it the system does not clearly exhibit any crystalline order (Gulik et al. 1985). However, on the basis of calorimetric and thermogravimetric studies (Gliozzi et al. unpublished work) it has been proposed that this transition corresponds to a change from a rigid to a flexible state of the hydrophobic chains which are still stacked in lamellae organized in rough surfaces, as sketched in Fig. 7. Indeed the relatively high value of the order parameter, after this transition has occurred, seems to support such a hypothesis. On the other hand a

rippled state of lamellae has been observed in the P_β phase of dipalmitoylphosphatidylcholine bilayers (Stamatoff et al. 1982). It is worthwhile to remark that the sudden decrease in the order parameter revealed by the 5SASL- and by the 12SASL-labelled systems, occurs around 35 °C. Therefore it is shifted by about 10 °C with respect to the above transition. Consequently, we are led to propose the occurrence of a new structural change involving the outer portion of the hydrophobic chain. Calorimetric measurements on the dry GDGT system have often revealed the occurrence of such a post-transition, exhibiting an enthalpy production five times smaller than that of the main transition (Gliozzi et al., unpublished work). In the hydrated sample, where the two transitions are much closer together, they are observed as a double peak (Miller et al. 1985). Furthermore, the order parameter has indicated a remarkable difference in the behaviour of the outer portion of the hydrophobic chains up to C_{12} with respect to the inner one. Thus it is not surprising that two different transitions involving two different portions of the same molecule might be found.

At higher temperatures ($T > 60$ °C) the order parameter increases far more rapidly for the 5SASL- and the 12SASL- than for the 16SASL-labelled system (cf. Fig. 3), indicating that segregation between polar and apolar regions tends to disappear. At $T > 70$ °C, the hyperfine splitting value $2A_\parallel$ of lecithin (cf. Table 1) becomes higher than that of GDGT, suggesting that the glycerol head groups of GDGT do not share the polar nature of normal monopolar lipids.

GDNT

The asymmetric lipid GDNT interacts with water to a much larger extent than the symmetric one. In fact, in the fully hydrated state about 20 water molecules bind to one lipid (Gliozzi et al. 1986). Consequently, at variance with GDGT, the GDNT molecular dynamics and structural organization is highly influenced by the degree of hydration, as shown by previous work (Gliozzi et al. 1983 b; Gulik et al. 1985; Miller et al. 1985). The present set of data exhibits changes in the trend of the measured parameters, exactly at those temperatures where phase transitions are known to occur. This fact provides direct evidence that the label introduces only minor changes in the organization of the sample.

A fundamental aspect of the asymmetric lipid behaviour relies on the slow motion of the external lipid portion, as revealed by ST-ESR. This is a consequence of the steric constraints imposed by hydrogen bonds between the nonitol polar head groups. Data reported in Table 1 indicate a much stronger immobilization of the outermost hydrophobic portion of GDNT, not only with respect to GDGT, but also when compared to a normal monopolar egg-lecithin lipid. It is worthwhile to notice however that 5SASL-labelled egg-lecithin and GDNT, both dry and hydrated, exhibit almost equal $2A_\parallel$ values at their physiological temperatures, i.e. at 37° and 85 °C, respectively. An additional feature to note is the quite similar value of $2A_\parallel$ in the internal hydrophobic acyclic chain portion of GDGT and GDNT for both dry and hydrated samples above 30 °C.

X-ray diffraction studies (Gulik et al. 1985) have indicated the occurrence of a broad structural change in the range 40–50 °C for dry GDNT, corresponding to a phase transition from a rectangular, P_α, to an hexagonal, H_{II}, phase. The plateau observed in L''/L and C'/C (Fig. 6) seems to be indicative of such a transition. The almost constant values of τ_c deduced from Fig. 6 could result from two counteracting contributions: a mobility increase induced by temperature and a decrease due to the P_α-H_{II} transition. The latter is suggested by the extrapolation of the C'/C curve below and above the transition range. On the other hand, the change in trend observed with the 16SASL at the same temperature (cf. Fig. 5 b) indicates the remarkable rearrangement of the deep hydrophobic core. Calorimetric experiments (Gliozzi et al. 1983 b) indicated the presence of a heat absorption peak in the same temperature range, characterized by an unusually small enthalpy production ($\cong 2$ J/g), probably related to changes in hydrogen bonds. Thus the dry state does not seem to imply the occurrence of stiff chains even at low temperatures (down to $T = 0$ °C), possibly because of the steric constraints imposed by the nonitol head groups.

In the fully hydrated state the rapid decrease in the 16SASL $2A_\parallel$ curve centred around 20 °C is in coincidence with the irregularity found in the 5SASL curve (which, although small, is outside of the experimental error and present in all the runs). X-ray diffraction data (Gulik et al. 1985) indicated at this temperature a transition from a rigid to an hexagonal H_{II} structure. The lower temperature phase (or phases) has not yet been identified. DSC measurements (Gliozzi et al. 1983 b) revealed an absorption peak of ~ 6 J/g, i.e. about 1/3 of the melting value of GDGT. Thus this transition is characterized by a smaller entropy variation with respect to the GDGT one ($\Delta S = 20$ mJ/g °K against $\Delta S = 60$ mJ/g °K for GDGT), owing to the steric constraints imposed by the polar heads. Nevertheless it seems to affect the inner as well as the outermost portion of the chain,

as indeed expected for the formation of an hexagonal structure.

The change in slope observed at 50 °C with the 5SASL sample corresponds to the transition from a pure hexagonal H_{II} phase to a composite H_{II} one with presumably water clusters (Gulik et al. 1985). This transition has also been revealed by a very small calorimetric peak ($\Delta H \gtrsim 1$ J/g) and by a break in the thermogravimetric curve (Gliozzi et al. 1986).

Biological considerations

The dynamic behaviour of the asymmetric lipid system is quite unusual: it indicates the ability of the nonitol polar heads to establish highly cooperative interactions even at high temperatures (~ 80 °C). Recalling that the nonitol polar head is exposed towards the outside of the cell (De Rosa et al. 1983b), such strong interactions might explain the great stability of the plasma membrane even under those extreme environmental conditions. Moreover, considering the very high pH gradient that the cell must withstand, such behaviour might play the physiological role of establishing, in addition to a mechanical one, an electrical barrier due to dipole-dipole interactions. By contrast the dynamic behaviour of the internal part of the lipid chain, at physiological temperatures (~ 85 °C) does not differ significantly from that of a normal lipid, like an egg-lecithin, at 37 °C. Considering that the mean value of the thickness of the acyclic part of the molecule is = 16 Å, such a portion may well provide the required fluid microenvironment necessary for functioning of proteins and enzymes.

It is known that when *Sulfolobus solfataricus* is grown at increasing temperatures, the number of cyclic rings of the lipid chain increases (De Rosa et al. 1980). The dramatic action of these cyclopentane rings on phase transition temperatures has already been stressed; indeed a single added ring is able to shift the transition of the dry asymmetric lipid by about 20 °C (Gliozzi et al. 1983b). The present ESR data underline a further aspect, indicating the completely different dynamic behaviour of the cyclic and acyclic portions of the same molecule. Thus the formation of rings might well be one of the evolutionary responses to the thermal fluctuations of the natural habitats of these archaebacteria.

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