Membrane Lipid Alkyl Chain Motional Dynamics Is Conserved in *Sarcina ventriculi* despite pH-Induced Adaptative Structural Modifications Including Alkyl Chain Tail to Tail Coupling†

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ABSTRACT: *Sarcina ventriculi*, an anaerobic Gram-positive bacterium, adapts to increasing temperature, the presence of organic solvents, or the lowering of the pH of its growth medium by joining the tails of membrane lipids from opposite sides of the bilayer, forming transmembrane, bifunctional fatty acid species. Since this is done to offset the increase in membrane mobility caused by these perturbations, it is of interest to determine whether the motional (dynamic) properties of membrane lipid alkyl chains are conserved. In this study, conservation of the motional time scales of the alkyl chains of total membrane lipids from *Sarcina ventriculi* cells grown at different pH values was demonstrated using proton nuclear magnetic resonance (NMR) spectroscopy. The NMR longitudinal relaxation times (T_1) of the protons in the bulk methylene groups were measured for lipids from cells grown at pH 3.0 and 7.0. These measurements indicated that the temperature profile of the T_1 relaxation behavior for the methylene protons from these two different preparations was the same. Analysis of the data from T_1 measurements indicated that the thermal barrier for relaxation is the same in both lipid systems. This is only true if the pH of the sample on which the measurement is being made is adjusted to the same value as that at which the corresponding cells were cultured. It is clear from this latter observation that the state of protonation of the lipid head groups is a contributor to the overall motional freedom of the membrane lipid components. The correlation times (τ_c) of characteristic lipid alkyl chain motion were estimated to be approximately 10^{-10} s. This study reaffirms the principle of homeoviscous adaptability and indicates that, during adaptation, conservation of structural features is secondary in importance to conservation of motional dynamics.

It is generally recognized that, to be functional, biological membranes need to be in the liquid crystalline phase. This is characterized by a complex mixture of domains, the physical and chemical properties of which are regulated by a large variety of interlinked chemical processes. Environmental changes may, however, shift the thermodynamic balance toward phases that are biologically nonfunctional. Membranes of bacteria, therefore, have evolved chemical mechanisms enabling them to maintain a dynamical state compatible with membrane functions in the face of adverse environmental changes. Membrane adaptative processes in bacteria are usually very dynamic, occurring even as the environmental conditions are being changed. Adaptative mechanisms that are triggered in response to temperature variations range from minor ones, such as modification of the chain length distribution of fatty acid residues and changes in the degree of fatty acid unsaturation (Cronan, 1975; Ingram, 1976), to more dramatic processes, such as synthesis of cyclohexane-containing fatty acids and synthesis of hopanes (Mycke et al., 1987; Ourisson, 1987). Recently, a physical interpretation of the significance of fatty acid heterogeneity in bacterial membranes has led to the idea that the lipid chains act as baths for a high density of energy states of magnitude kT. It was further proposed that this distribution of states varied to reflect the Maxwellian distribution of kinetic energies characteristic of that temperature (Hollingsworth, 1995).

Sometimes the environmental perturbations are too large to be met by simple changes in fatty acid chain length and require more extreme structural changes. One especially dramatic adaptative response has recently been uncovered in *Sarcina ventriculi*. The discovery of this response began with the characterization of unusual, very long α,ω-bifunctional fatty acids in this organism when it was subjected to increasing temperature, treatment with organic solvents, or depression of pH (Jung et al., 1993). It has subsequently been demonstrated using combinatorial arguments (Jung & Hollingsworth, 1995) and chemical arguments (Jung & Hollingsworth, 1994), that these α,ω-bifunctional fatty acids might be formed by rapid, dynamic, tail to tail coupling of existing fatty acids from opposite sides of the membrane bilayer (Figure 1). More recently, a cell-free activity capable of taking foreign, exogenously added fatty acids that were incorporated into membrane vesicles and further incorporating them (tail to tail) into existing mem-

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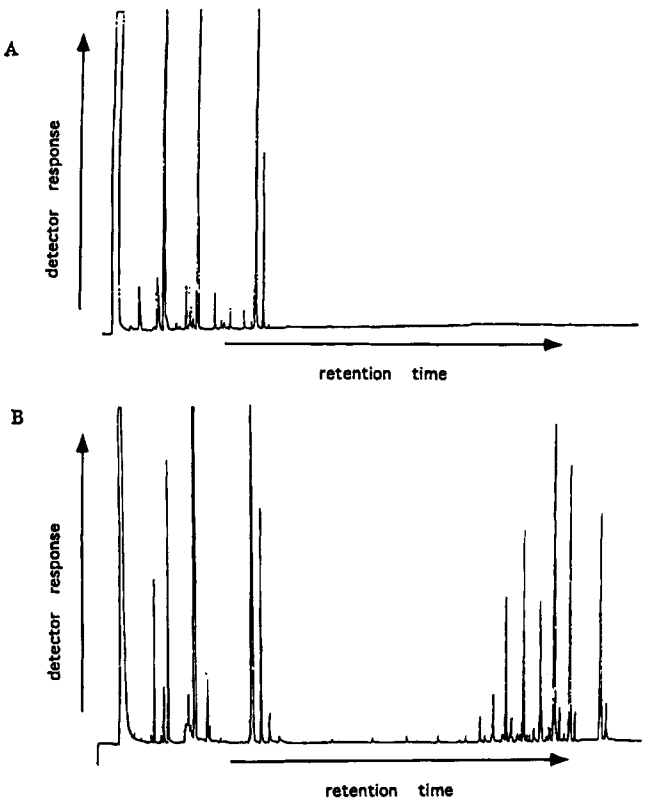

FIGURE 1: Gas chromatogram of lipid components from the membrane of *S. ventriculi* analyzed as fatty acid methyl ester derivatives: (A) fatty acid methyl esters from cells grown at pH 7.0 and (B) from cells grown at pH 3.0.

brane lipids has been demonstrated (Lee et al., 1994). This process has some general significance among eubacteria, since very long α,ω-bifunctional fatty acids have been found in several other eubacteria including *Thermoanaerobacter ethanolicus* (Jung & Hollingsworth, 1994), *Butyrivibrio* sp. (Clarke et al., 1980; Hazelwood & Dawson, 1979; Klein et al., 1979), and *Thermatoga maritima* (Huber et al., 1986). In *Butyrivibrio*, tail to tail coupling has also been suggested (Clarke et al., 1980). It has also been demonstrated that, in archaebacteria, the proportion of tetraether lipids, now thought to be formed by transmembrane tail to tail coupling of normal diethers (Kushwaha et al., 1981; Nishihara et al., 1989), is increased with increasing temperature (Sprott et al., 1991).

From the standpoint of molecular dynamics, it seems intuitively reasonable that one way of offsetting the increased membrane motion caused by (say) a temperature increase or the addition of an organic solvent or the lowering of pH (which decreases membrane organization by reducing the negative charge of the head groups, thus reducing the extent of their cross-linking with cations) is to tie the tails of fatty acid groups from opposite sides of the membrane together. The extent of this and other supporting structural modifications should just balance the magnitude of the perturbation. This is, in fact, a statement of the principle of homeoviscous adaptability (Sinensky, 1974) in molecular terms. In a more general sense, the idea of dynamic regulation of molecular events by living organisms through dynamic feedback can be advanced. The obvious question to ask is the following: "What motional (dynamic) aspect of membrane character has been conserved during this adaptative response, and how can this be measured?" One good candidate for such a measurement is the motion of the alkyl chains of the membrane lipids. This is reasonable since one outcome of this striking adaptative response should be to conserve the average motional (dynamic) properties of the bulk membrane lipid chains, thus ensuring that the catalytic properties of membrane proteins (which are controlled by viscosity) and the frequencies of events such as channel openings and closings are not impaired.

Nuclear magnetic resonance (NMR)[1] spectroscopy is an excellent practical tool for measuring the dynamic properties of molecules of all sizes. One especially useful NMR parameter is the spin-lattice or longitudinal relaxation time, T_1. This parameter measures the rate of reestablishment of the equilibrium magnetization of the spins (nuclei) in question after it has been disturbed by the absorption of a radiofrequency pulse. The rate of recovery of magnetization is a measure of the motional freedom of the groups in question (Abragam, 1961). In any NMR experiment, the measuring pulse or the preceding pulses cause a departure from the Boltzmann distribution of spin states. Before the next acquisition, the Boltzmann population of spin states must be reestablished. For spin 1/2 nuclei (such as protons), the return to this distribution occurs exponentially with a rate constant of $1/T_1$, where T_1 is the spin-lattice (or longitudinal) relaxation time. The fundamental origin of this relaxation phenomenon is energy exchange by dipole−dipole interactions or exchanges, the frequency of which is determined by the extent of motion of the nuclei in question.

Information on the time scale of the motion of the relaxing nuclei can also be obtained from a determination of the probability that the vector separating two dipoles which are generating relaxing oscillating fields remains oriented relative to some fixed direction. This probability decreases exponentially with time with a time constant of magnitude τ_c. This value, τ_c, is also called the molecular correlation time and is a good measure of the time scale of motion of the relaxing nuclei and can be related to T_1. Hence, the longitudinal relaxation time for a spin 1/2 nucleus or group of nuclei is related to the correlation time (τ_c) of their molecular motion by the relation:

$$1/T_1 \propto \tau_c/1 + (\omega_o\tau_c)^2 \qquad (1)$$

where ω_o is the Larmor frequency in radians/second. It follows from eq 1 that, for T_1 to be a minimum, the time scale of molecular motion (τ_c) must be matched to the spectrometer frequency, such that the condition $\omega_o\tau_c \approx 1$ (actually $\omega_o\tau_c = 0.7$) is met at a particular temperature. At this condition, the relaxation time is a minimum and the relaxation mechanism is most efficient. Temperatures giving rise to motional time scales greater or less than τ_c lead to a reduction in relaxation efficiency and an increase in T_1. For appropriate spectrometer frequencies, therefore, a plot of T_1 vs temperature (or 1/temperature) passes through a minimum. It is then possible to determine τ_c at this temperature since it is approximately equal to $1/\omega_o$. The theory tested in this study is that after a perturbation that leads to adaptative tail to tail coupling (along with any other head group modifications) the motional dynamics of the bulk methylene group is still unchanged at any temperature, resulting in τ_c being the same at the same temperature in both cases.

[1] Abbreviations: NMR, nuclear magnetic resonance.

MATERIALS AND METHODS

Organism and Culture Conditions. *S. ventriculi* JK was cultivated as described previously (Goodwin & Zeikus, 1987). Growth under pH control was performed using a 12 L Microferm fermentor (New Brunswick Scientific, Edison, N.J). The fementor was equipped with a pH electrode, and the pH was adjusted by the addition of either 5 M NaOH or HCl. Cells were harvested at midexponential phase, washed with distilled water, and stored at −20 °C.

Total Fatty Acid Analysis. Fatty acid analyses were performed on whole cells as described previously (Jung et al., 1993), with slight modifications. Briefly, approximately 5 mg (wet weight) of cells was suspended in 0.3 mL of chloroform and 1.5 mL of 5% methanolic hydrochloride solution and heated for 24 h at 72 °C. The mixture was sonicated (5 min) every 8 h. The samples were dried under nitrogen and partitioned between water and chloroform. The organic phase was filtered through glass wool. The fatty acid methyl esters so prepared were subjected to gas chromatography analysis on a 25 M J&W Scientific DB1 capillary column, using helium as the carrier gas, a temperature program of 150 °C initial temperature and 0.0 min hold time, and a rate of 3 °C/min to a final temperature of 300 °C. This temperature was held for 70 min.

T_1 Measurements. Proton T_1 measurements were performed at 300, 400, and 500 MHz by the inversion recovery technique using a $\pi-\tau-\pi/2$ pulse sequence. Lipids for T_1 measurements were prepared by drying 5 mg of total lipid extract under nitrogen gas followed by evacuation under low pressure for at least 8 h to ensure complete removal of chloroform. The lipids were then resuspended in 0.7 mL of phosphate buffer saline (PBS) (Dulbecco) made up in deuterated water. The resulting suspension was sealed under nitrogen, heated to 55 °C for 2 min, and cooled to room temperature. This cycle was repeated five times to ensure good hydration. The sample was then transferred to a 5 mm NMR tube and sealed under nitrogen gas.

RESULTS AND DISCUSSION

The presence and high proportion (>50%) of very long bifunctional fatty acids in the cells of *S. ventriculi* grown at pH 3.0 have been well documented (Jung et al. 1993, 1994) and are reproduced here for completeness (Figure 1) by gas chromatographic analysis. These fatty acids were shown by mass spectrometry in the last cited works to possess alkyl chains of up to 36 carbons. The proportion of long chain lipids relative to regular chain lipids (14−18 carbons) in cells grown at pH 3.0 was estimated by integrating the peak areas of the GC analysis and found to be in excess of 50%. In contrast, cells grown at pH 7.0 have none or only a small proportion of long chain lipids (<10%). Freeze fracture electron microscopy of membranes from cells grown at pH 3.0 did not show any evidence of concave/convex fracture between the bilayer leaflets, suggesting that the long chain lipids span the entire membrane thickness (Jung and Hollingsworth, unpublished results). The resulting lipid arrangement is, therefore, that of a bipolar monolayer. A schematic representation of a membrane system containing long chain lipids is shown in Figure 2.

The 500 MHz proton NMR spectra of lipid extracts from cells of *S. ventriculi* grown at pH 7.0 or 3.0 in aqueous suspensions (in buffer at pH 7.0 and 3.0, respectively) are

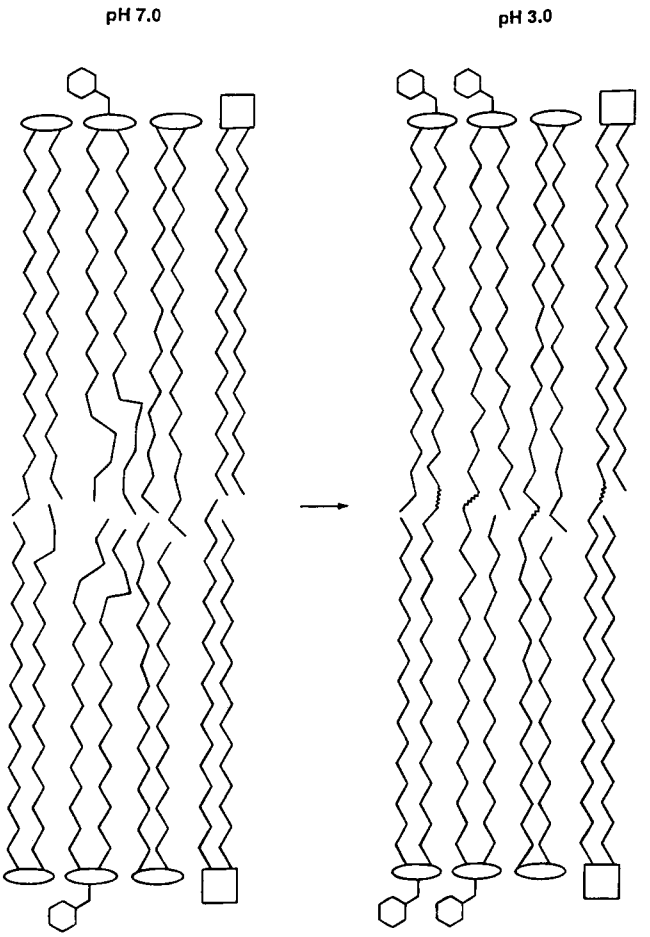

FIGURE 2: Schematic diagram of a membrane arrangement from *S. ventriculi* grown at pH 7.0 or 3.0. Note the long chain lipids at pH 3.0 with tail to tail coupling represented by wavy lines.

shown in Figures 3A,B respectively. The broad lines, which result from dipolar coupling, are typical of aqueous lipid suspensions. The strong signal at approximately 1.5 ppm was assigned to the bulk of the methylene protons of the acyl chain and the signal at 1.0 ppm to the terminal methyl protons. Because the two resonances were well resolved, it was possible to probe the motion of both the terminal part and the bulk of the alkyl chain by measuring T_1 relaxation times for the methyl and methylene protons. Typically, the recovery of the equilibrium magnetization as a function of time τ after perturbation by a π pulse followed exponential behavior, as shown in Figure 4A. T_1 values for methyl and methylene protons obtained at 500 MHz and were plotted as a function of temperature and are shown in Figure 4B. A global T_1 minimum was observed for the methylene protons of lipids from cells grown at pH 7.0 and 3.0 and for the methyl protons of lipids from cells grown at pH 3.0, when the pH of the sample was the same as the pH at which the cells were grown. These global minima appeared at approximately the same temperature (around 20 °C). As was stated earlier at the temperature of the minimum, τ_c can be evaluated, and at the proton frequency of 500 MHz used here ($\omega_o = 2\pi \times 500$ MHz) τ_c is $\approx 10^{-10}$ s. T_1 was also measured at 300 MHz (Figure 5). Only the methylene proton relaxation times are reported here. At this frequency no global minimum was observed. The absence of such a minimum at lower field indicated that the one observed in the 500 MHz experiment was not due to a freezing

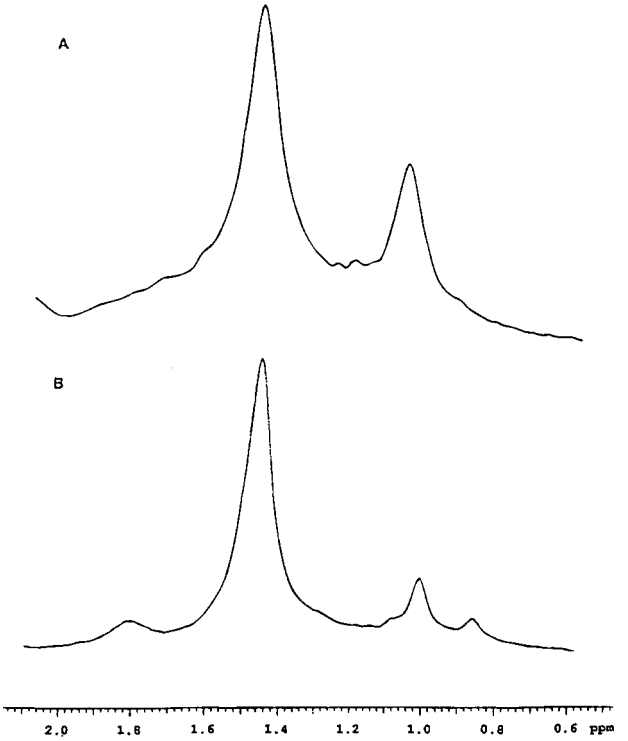

FIGURE 3: 500 MHz ^{1}H NMR spectra of total lipid extract from *S. ventriculi* grown at pH 7.0 (A) and 3.0 (B) in aqueous suspension (in PBS at pH 7.0 and 3.0, respectively). Spectra were obtained at 40 °C.

phenomenon or some other such artifact. The minimum is expected to appear at a lower temperature at lower field strength. At the field corresponding to 300 MHz, the minimum appears to be just outside of the temperature range used for the study. A plot of $1/T_1$ vs ω^{-2} (for fields corresponding to 300, 400 and 500 MHz for protons) at any particular temperature did give a straight line as is expected for nuclei undergoing relaxation by a purely exponential process (Figure 6).

The temperature dependence T_1 curves at 500 MHz also showed evidence of the presence of unresolved local minima. These could be attributed either to separate domains that had the same correlation time (but at different temperatures) or to a dynamic shifting of membrane structure as a function of temperature, such that the correlation time passes through that same value over a range of temperatures. Both possibilities are especially interesting since it means that, at any given temperature, there will always be domains such that critical membrane proteins can always be in the same dynamic environment. The presence of these local minima at different temperatures (suggesting domains with the same correlation time at different temperatures) also means that local temperature fluctuations will be dynamically buffered since the same dynamic state is reproduced several times along the changing temperature profile.

The fact that the methyl groups in lipids obtained from cells grown at pH 3.0 and measured at pH 3.0 also have dynamics similar to those of the bulk methylene groups is not too surprising since their motion will be constrained by neighboring tail to tail coupled alkyl chains with very limited mobility. As expected, the behavior of the terminal methyl groups of the lipid chains of cells grown at pH 7.0, thus containing little or no transmembrane lipid species, was markedly different. There was no global minimum, but there

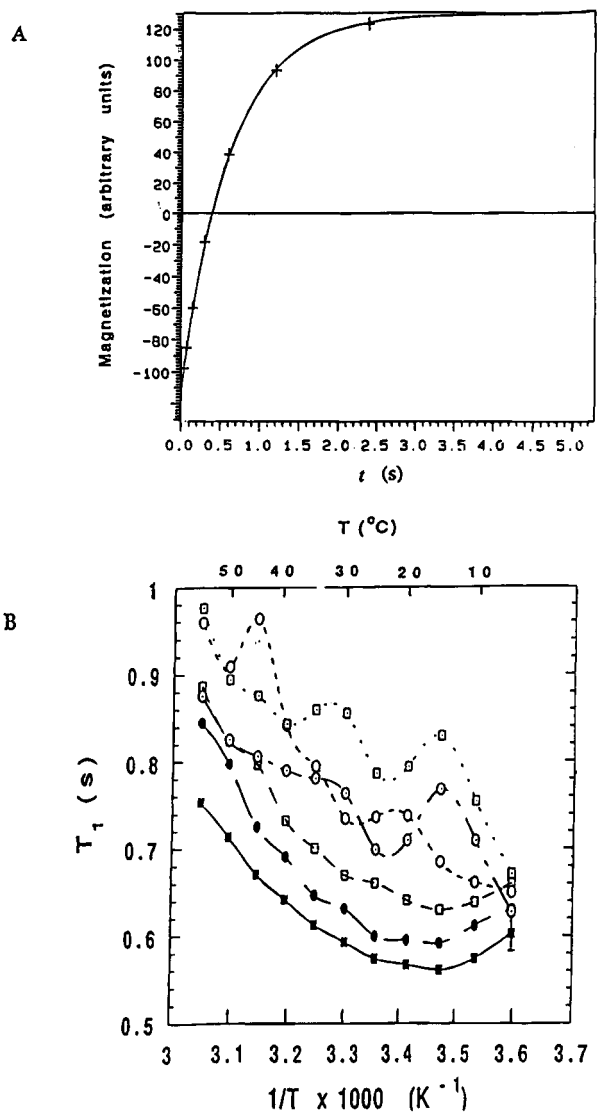

FIGURE 4: (A) Typical recovery of the magnetization $M(z)$ of the methylene groups as a function of time after inversion. (B) 500 MHz T_1 spin-lattice relaxation time for methylene (squares) and methyl (circles) protons of alkyl chains as a function of inverse temperature. Open symbols represent lipid extract obtained from cells of *Sarcina ventriculi* grown at pH 7.0 in suspension in aqueous buffer (pH 7.0). Closed symbols represent lipid extract from cells of *S. ventriculi* grown at pH 3.0 in suspension in aqueous buffer (pH 3.0), and open symbols with dots represent lipid extract from cells of *S. ventriculi* grown at pH 7.0 but in suspension in aqueous buffer at pH 5.0. Error bars are shown only for the closed squares and represent the standard error from the regression analysis on the magnetization $M(z)$ vs time (from the pulse sequence) used to determine T_1. Typically the error bars did not exceed the size of the symbols. Each curve corresponds to one experiment representative of several repeats. The absolute value for T_1 is within 10% from sample to sample. However, the local and global minima did not change.

were several pronounced local minima with much longer relaxation times, indicating relatively free unrestricted motion.

It is our hypothesis that a particular membrane lipid chemistry resulting from bacterial adaptation will support optimum membrane dynamics only in environmental conditions equivalent to those under which adaptation occured. We therefore predicted that, by suspending lipid extracts in buffer at a different pH than that at which cells were grown, lipid alkyl chain dynamics should be perturbed. Indeed,

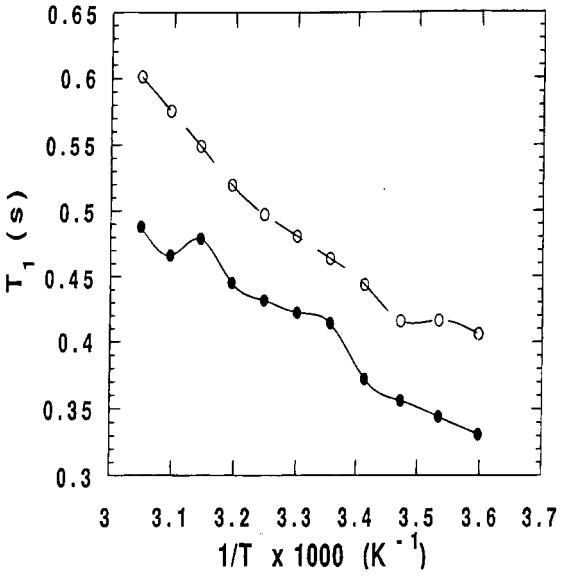

FIGURE 5: 300 MHz T_1 spin-lattice relaxation time for methylene protons of alkyl chains as a function of inverse temperature. Open symbols represent lipid extracts obtained from cells of *S. ventriculi* grown at pH 7.0 in suspension in aqueous buffer at pH 7.0. Closed symbols represent lipid extract from cells of *S. ventriculi* grown at pH 3.0 in suspension in aqueous buffer at pH 3.0.

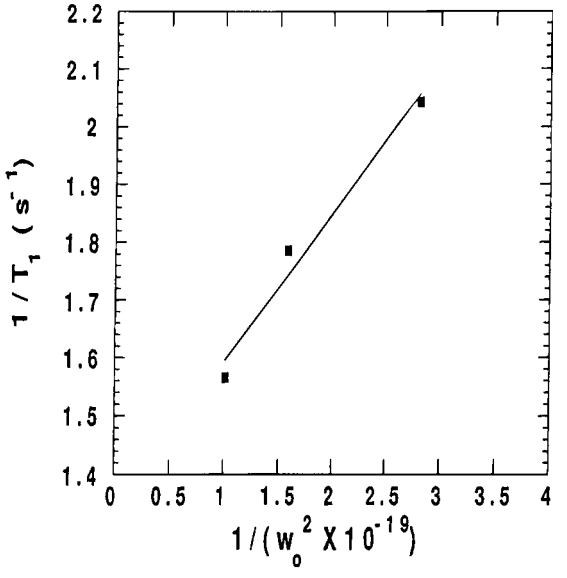

FIGURE 6: Frequency dependence of T_1 at 37 °C.

measurements at pH 5.0 of the temperature dependence of T_1 relaxation times of lipids isolated from cells cultured at pH 7.0 resulted in a profile very different from that obtained when the measurements were made at pH 7.0 (Figure 4B). These measurements were not made for the reverse condition (low-pH-grown cells measured at higher pH) because such systems were unstable and tended to precipitate. Since pH can hardly affect the motion of alkyl chains directly, this perturbation is most likely being transmitted through the protonation and charge state of the head group. This is reasonable since the lipid head group charge state determines the extent of their solvation and their interaction with and cross-linking by metal cations (Cevc, 1990). This, in turn, determines the stability of the membrane structure and the extent to which individual lipid molecules can diffuse in the plane of the membrane. The extent of lateral motion and the space between lipid head groups do affect the packing density of the hydrocarbon chains (Cevc, 1987). Structural modifications involving the preservation of alkyl chain dynamics should, therefore, involve some lipid head group contributions. In earlier work (Lee et al., 1994), we demonstrated that the adaptation of *S. ventriculi* to low pH also involves important head group structural modifications. These modifications included the head to head coupling of lipid molecules via acetal linkages. This mechanism leads to the formation of a family of structures in which various head group components are modified by acetalization to other components.

In order to better understand the nature of the molecular motions giving rise to T_1 relaxation in our lipid systems, we used NMR relaxation theory together with the model proposed by Petersen and Chan (1977), which provides a framework to explain the T_1 behavior of protons in lipid alkyl chains. For spin 1/2 nuclei, relaxation by dipole-dipole interaction is usually dominant. It has been demonstrated that the methylene protons relax by interacting with other methylene protons in the same alkyl chain instead of with those in neighboring chains (Kroon et al., 1976). Intramolecular dipolar spin-lattice relaxation for a two-spin system with equal spins can be described (James, 1975) by the relationship:

$$1/T_1 = 2\gamma^4\hbar^2 I(I+1)/5r^6((\tau_c/1 + \tau_c 2\omega_o 2) + (4\tau_c/1 + 4\tau_c 2\omega_o 2)) \quad (2)$$

where γ is the magnetogyric ratio, $\hbar$ is the Planck constant divided by 2π, I is the quantum number, and r is the distance separating the two interacting nuclear spins. If relaxation is an exponential process, as we show in Figure 4B, then the relaxation frequency (υ) can be related to the energy barrier by the relationship

$$\nu = \nu_0 \exp(-E_a/kT) \quad (3)$$

where E_a is the activation barrier characteristic for the relaxation process and k is the Boltzmann constant. This allows one to write the customary relationship:

$$\tau_c = \tau_o \exp(E_a/RT) \quad (4)$$

The activation process in membrane alkyl chain relaxation is known to be governed by *gauche−trans* isomerization, and, for normal bilayer membranes, the value of E_a has been estimated to be 3−4 kcal/ mol (Petersen & Chan, 1977). Hence, by knowing E_a (which is a constant) and τ_c at any one temperature, it was possible to evaluate the second constant τ_o in eq 4. These values could then be used to evaluate τ_c at any other temperature by substitution into the same equation. Once τ_c (at any given temperature) is known, one can then calculate T_1 from eq 2. This would, however, require evaluation of the term $2\gamma^4\hbar^2 I(I+1)/5r^6$ in that equation. The only potential variable in this term is r which is the distance separating the relaxing dipoles in the same chain and is very temperature insensitive. The entire term can, therefore, be evaluated and treated as being independent of temperature.

Since the plot of T_1 vs 1/T passes through a minimum at a field strength corresponding to 500 MHz, ω_o, T_1, and τ_c are all known under this special condition. The term in r^6 could then be calculated. The calculation from eq 2 of T_1

Table 1: Parameters for the Theoretical Fitting of T_1 Data[a]

temperature (K)	τ_c ($\times 10^{-10}$ s)	T_1 (pH 3.0) (s)	T_1 (pH 7.0) (s)
278	2.97	0.59	0.67
283	2.53	0.57	0.65
288	2.19	0.56	0.63
293	1.91	0.56	0.63
298	1.67	0.57	0.64
303	1.47	0.58	0.65
308	1.30	0.60	0.67
313	1.15	0.63	0.71
318	1.03	0.66	0.75
323	0.92	0.70	0.79
328	0.82	0.75	0.85

[a] The error on T1 values was estimated to be between 5 and 10%.

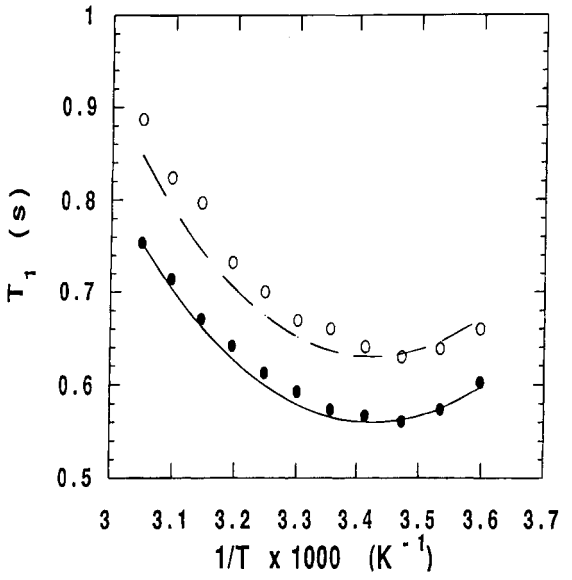

FIGURE 7: Theoretical fit of the methylene proton T_1 values using eq 2 and values from Table 1. Solid lines represent the calculated values and symbols are the experimental data points reproduced from Figure 4, at pH 3.0 (circles) and 7.0 (squares) lipids.

at any temperature, using τ_c values calculated from eq 4 with a value for E_a of 4.6 (obtained by trial but based on the original estimate of 3−4 by Petersen and Chan), was then possible. We evaluated T_1 as a function of temperature for the methylene protons of pH 7.0 and 3.0 lipids (Table 1). The calculated curves (Figure 7) followed the envelope of the measured curves and displayed a minimum at the same position, indicating that these unusual membranes had the classical relaxation energetics. The T_1 values at the minima for the two curves were not exactly the same (though very similar), but this only reflected the fact that the membranes are structurally very different. The fact that they passed through a minimum at the same temperature, however, was more important and confirmed that the correlation times for their relaxation are the same at that temperature (Figure 7). The calculations also confirmed that even these highly cross-linked membrane systems (at pH 3) had the same dynamics and activation energy as the classical membrane systems. The Petersen−Chan model predicts a τ_c of approximately 10^{-10}−10^{-11} s, which is in very good agreement with our estimates. A similar τ_c of $\approx 10^{-10}$ s for *gauche−trans* isomerism in deuterated lipids was observed by Meier et al. (1986) and Seelig et al. (1981). The fact that both the activation energy and τ_c are very similar for membrane lipid alkyl chains of pH 7.0 and 3.0 cells strongly suggests that the average number of *gauche* conformers, and their rate of propagation along the chain axis (Horowitz et al., 1972; Peterson & Chan, 1977), is the primary aspect of membrane dynamics that is being conserved during this adaptation.

CONCLUSION

Results obtained in the present study indicate that, despite dramatic changes in the structure of the membrane lipids, the molecular motion of membrane lipid alkyl chains of *S. ventriculi*, detected by spin-lattice relaxation time measurements, is conserved upon changing the growth pH from 7.0 to 3.0. At 500 MHz, it was possible to estimate the correlation time (τ_c) of the relaxation process at the temperature of the global minimum. Membrane lipids are shown to be dynamic with respect to their chemical composition. This dynamically regulated composition ultimately ensures that some important aspects of the molecular motion of the lipid molecules remain constant with changing environmental parameters in order to support function. In this study, we have demonstrated that the activation barrier attributed to kink diffusion and *gauche−trans* isomerization is conserved. This is an important aspect of molecular function. It has been proposed that the thermal energy trapped in collective vibrational modes of membranes are dynamically tuned to the magnitude of the thermal energy bath, kT (Hollingsworth, 1995). The theory of homeoviscous adaptation also indicates that organisms can dynamically regulate membrane structure to conserve molecular function. Taken together with the results described here, it can be concluded that this dynamic regulation of membrane structure and molecular motion is a critical property of living systems.

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