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THE KINETICS OF THE FORMATION OF ROTATIONAL ISOMERS IN THE HYDROPHOBIC TAIL REGION OF PHOSPHOLIPID BILAYERS *

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Summary

Very fast structural changes in dipalmitoyl phosphatidylcholine molecules forming a vesicular bilayer were investigated by means of a laser temperature-jump technique. After temperature increases of about 1 K within 1 ns, the solution turbidity increases with a time constant of about 4 ns. This time constant exhibited no appreciable temperature dependence and represents a noncooperative process. It is interpreted as a local increase in density in the bilayer which results from a shortening of the individual lipid molecule due to formation of rotational isomers (e.g., kinks) without an appropriate expansion of the molecular environment. The final membrane expansion is achieved in consecutive steps with a decrease in turbidity and time constants between 100 μs and several seconds which are maximal in the midpoint of the phospholipid phase transition. These steps represent cooperative processes, namely the molecular interaction leading to the membrane expansion. The rate of kink formation implies that kinks migrate through the membrane by energetic transitions forwarded from lipid to lipid, rather than by hopping of individual lipids thereby carrying a kink.

Introduction

Turbidity or scattered-light intensity is frequently applied as a means of observing the phase transitions of one-component lipid bilayers [1,2]. These

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methods yield relatively nonspecific quantities in terms of structural information, but they offer the advantages of detection without the use of labels and of easy applicability in kinetic experiments which are essential for the elucidation of the transition mechanism.

Time-resolved changes of turbidity after a perturbation of the state of the bilayer by a temperature jump [3] and a pressure jump [4] reveal the existence of at least three effects which are clearly distinguishable and separated on the time scale. Two such effects were characterized in the range between 100 μs and several seconds. They were found to have cooperative properties expressed mainly by time constants of maximal magnitude at the midpoint of transition and of sharply dropping magnitude above and below the midpoint. The fastest effect, however, was completed within the dead-times of the classical temperature- as well as pressure-jump methods, i.e., within less than 5 μs after the perturbation of the equilibrium.

Very few results of kinetic experiments on lipid bilayers which are potentially suitable for the time resolution of effects occurring in the nanosecond time range have been published so far, among these are frequency-dependent absorptions of ultrasound [5—8].

It was found in all cases that the ultrasonic absorption was maximal at or near the midpoint of the phase transition. Since the excess absorption is described as a function of frequency by:

$$\alpha\lambda = \sum_i A_i \frac{\omega\tau_i}{1 + (\omega\tau_i)^2} \tag{1}$$

where α is the attenuation coefficient, λ the wavelength of sound, ω the circular frequency, A_i the relaxation amplitude and τ_i the relaxation time of process i. This maximum could be caused by the amplitude A_i or the time constant τ_i, or both simultaneously. Therefore, it is not permissible to decide about cooperativity properties of the investigated process before the individual contributions of A_i and τ_i to the overall absorption are known. This is especially so because A_i exhibits a maximum at the midpoint of all isomeric equilibria, whether or not they are cooperative. Hence, our intention had to be to characterize the temperature dependence of the relaxation time τ, or times τ_i in case more than one process contributes to the chemical relaxation. Two reasons appear to be responsible for the fact that in only one case [7] could the authors characterize relaxation times and amplitudes: firstly, ultrasonic relaxation exhibits more than one process [6,8] and secondly, the requirement of a broad frequency range is a serious experimental restriction.

Jump-relaxation methods yield more direct access to the relevant quantities which make the application of our recently developed laser temperature-jump technique to the kinetic study of lipid phase transitions an attractive feature [9—11]. Contrary to all other jump techniques, the overall time resolution of this method (minimum rise time less than or equal to 1 ns, independent of additive) allows the observation of processes which are otherwise only accessible by stationary methods or are not measurable at all. Moreover, we consider it an advantage that the mode of detection, turbidity, can be chosen to be the same in this technique as in the jump experiments concerned with the millisecond effects.

Experimental Procedure

Lipids

The lipid, L-β,γ-dipalmitoyl-α-phosphatidylcholine, was purchased from Fluka, Switzerland, and used without further purification.

Preparation of lipid aggregates

Vesicles (one-shell aggregates) of dipalmitoyl phosphatidylcholine were prepared by injecting 25.9 mM ethanolic lipid solution into the buffer at a temperature (50°C) above the phase transition temperature (40°C), thereby creating an ethanol concentration of 7.5% (v/v) in the buffer. After injection, the solution was dialysed against pure buffer for at least 8 h [13]. The buffer consisted of 10 mM Tris-HCl (pH 7.5 at 25°C), 100 mM NaCl and 1 mM NaN_3. Under these conditions, we obtained vesicles with an outer diameter of 60 nm which was measured by means of quasi-elastic light scattering.

Laser temperature jump

An ultraviolet flash-induced dissociation reaction of the gas, perfluoroisopropyl iodide (i-C_3F_7I) into $C_3F_7{\cdot}$* and I * ($^2P_{1/2}$) is used for laser action (transition between I * ($^2P_{1/2}$) and I ($^2P_{3/2}$)). In detail, this iodine laser has been described previously [9—11]. From the emitted pulse train at 1.315 μm a single pulse is selected, amplified and passed through a quartz cuvette with the aqueous solution under investigation, resulting in a laser pulse half-width of 0.6—3 ns depending on the mode of operation. The laser light energy is absorbed by rotational-vibrational states of the water molecules (the solvent) and converted into heat within a time equal to the duration of the laser pulse. This is due to the very short life-times of excited vibrational-rotational states in H_2O [14]. The conversion of energy results in a temperature jump of about 1 K within an effective volume of approx. 40 μl in a quartz cuvette containing 500 μl sample solution. The cuvette was thermostatically controlled with an accuracy of ±0.1 K. Laser temperature jumps were repeated about every 3 min within which thermal equilibrium of the cuvette was reached.

Perpendicular to the laser light beam, the intensity attenuation of 365 nm light from a xenon lamp (XBO 150 W) was detected. The lamp was pulsed by discharing a capacitor bench of 60—80 V within 500 μs. This made possible the use of a very fast low-noise photomultiplier [15] with a rise time of 0.6 ns which operates with only five dynodes and therefore requires high lamp power. This lamp pulse is induced simultaneously with the laser emission. Filters before and a monochromator behind the sample ensured that only the desired part of the detection light passed through the sample and was detected by the photomultiplier tube (RCA 1P28). The signal was passed through an active probe (Tektronix P6201, bandwidth 900 MHz) and stored in a Tektronix transient digitizer 7912 AD equipped with plug-ins 7A 13 and 7 B 92 (bandwidth greater than 100 MHz, rise time 2.6 ns. The Tektronix 7912 AD was connected to a calculator (Hewlett-Packard 9845 S) via an IEEE 488 bus. Several experiments were averaged at each temperature by means of the above-mentioned calculator.

Results

Relaxation curves were obtained for the temperature range of 32—43°C including the dipalmitoyl phosphatidylcholine phase transition temperature, $T_m = 40°C$. The relaxation proceeds with an increase in solution turbidity within the investigated time interval. The relaxation time of this effect was determined as 4 ± 0.5 ns without significant dependence on temperature, especially without a pronounced maximum at the transition midpoint (40°C).

In Fig. 1, a single relaxation curve and an average over four signals are given. A least-squares fit of the signals between 3 and 20 ns results in a relaxation time of 5 ± 0.5 ns. If we take into account the influence of the electronic detection circuit, we calculate the real relaxation time as 4 ± 0.5 ns. At the phase transition temperature we find a small increase in the amplitude of 10—20%, but this result is not pronounced enough to be reliable. Further experiments are necessary to determine how much the amplitude changes at T_m.

The characteristics of the relaxation times and amplitudes are quite contrary to those of the respective slower microsecond and millisecond effects [4]. The sign of the amplitudes is positive (increasing turbidity with increasing temperature) and the dependence of relaxation times on temperature exhibits the characteristics of noncooperativity. These points are essential for the design of our model which is presented in the following section.

Discussion

The present results, in combination with those from ultrasonic absorption on lipid bilayers as obtained by a number of other authors [5—8], allow the partial design of a model for the phase transition of lipid membranes. In the following this will be introduced and discussed in the light of the available experimental data.

Our model is depicted schematically in Fig. 2. It is assumed that the phase transition proceeds in a sequence of steps. On raising the temperature (or lowering the pressure), at first a structural change is induced in the chains of a number of lipid molecules. This process is accomplished within at most 10 ns and represents the formation of rotational isomers (e.g., kinks, as introduced by Träuble and Haynes [16] and Seelig and Seelig [17]. It has a maximal prob-

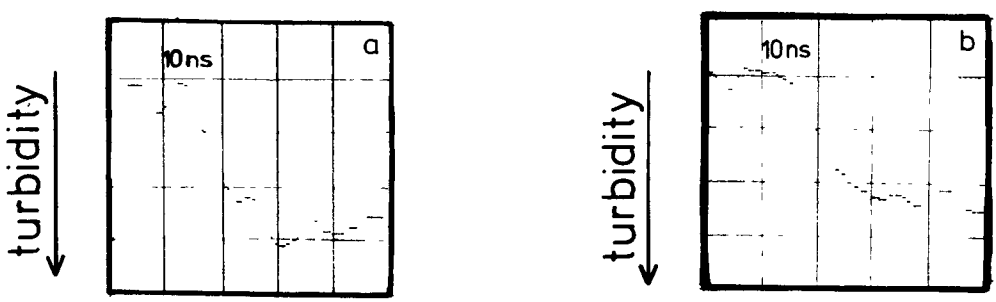

Fig. 1. (a) Turbidity (increase) relaxation of dipalmitoyl phosphatidylcholine vesicles at 43°C; single experiment. The grid unit corresponds to 10 ns. (b) Same specifications as for a, but four experiments were superimposed and averaged.

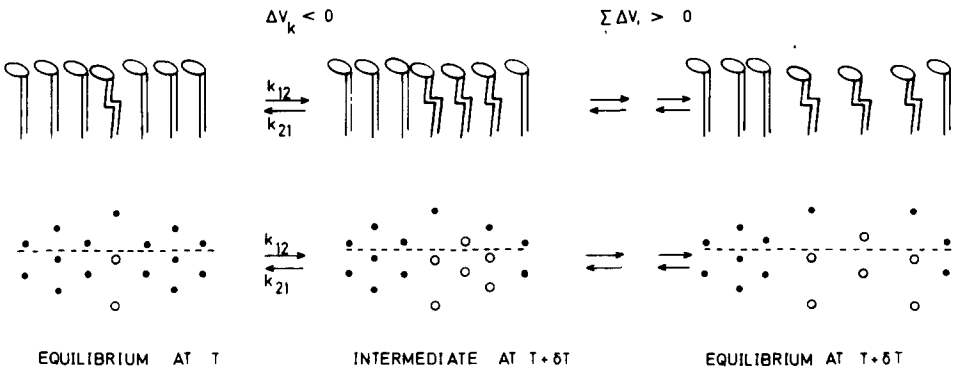

Fig. 2. Schematic arrangements of lipid molecules in the hexagonal lattice of a bilayer. •, molecules in the solid state; ○, molecules in the fluid state. From left to right the scheme depicts the step-wise assumption of a new equilibrium state induced by a rise of temperature. The broken line represents a vertical cut through the bilayer. The molecules bordering this cut are shown with the appropriate structure above the lattice.

ability of occurrence in the midpoint of the transition. The time of 10 ns does not suffice, however, to bring about the new equilibrium state of the membrane. The completion of the new state requires at least an expansion of the membrane to a larger surface area per molecule, a smaller bilayer thickness and an increase in the internal vesicular volume surrounded by the membrane [18—20]. The steps between the formation of rotational isomers and the completion of the new equilibrium include cooperative processes on a much longer time scale than the initial 'kink-step' [3,4,21]. The nature of these slower steps, however, will not be discussed here.

The observed fast effects are concentration independent. That they are intramolecular processes rather than rearrangements within the aggregate is plausible for two reasons. First, we did not detect any dependence of relaxation times on the type of the lipid aggregate (vesicle or liposome), contrary to the microsecond and millisecond effects [4]. Second, even a dramatic change of lipid headgroup structure such as the introduction of a charge, as done by Hammes and Roberts [5] by measuring ultrasonic absorption on phosphatidylserine, does not change the time range of relaxation.

We find our experimental results and interpretation reconfirmed by the fact that the correlation time for the rotational motion of lipid chain segments was measured to be between 10 and 100 ps by ^{2}H-NMR [22]. It should be noted, however, that turbidity detects an overall process and is not specific. Changes in the location of the head groups which occur on the same time scale as our turbidity changes may also contribute to our signal [23—27], but are not inferred in our model.

The formation of a kink within a time interval in which the environment of the converting molecule cannot accommodate the need for the required space implies a local shrinkage of the bilayer thickness and a simultaneous increase in density. This agrees with the observation of a turbidity increase after the fast temperature jump, since turbidity is proportional to the fourth power (for point scatterers) of the refractive index, n, which in turn increases linearly with density [1]. Another source of increasing turbidity could be an increase in

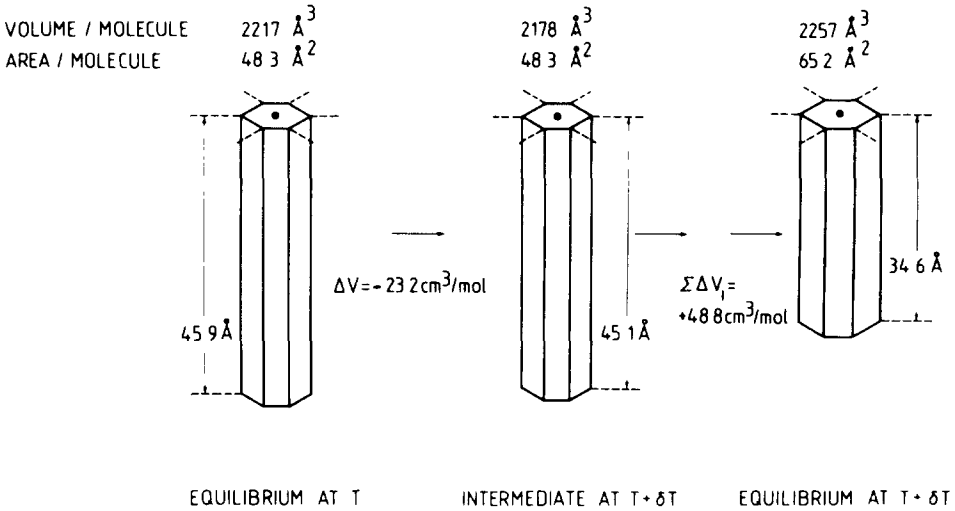

Fig. 3. Schemes of the molecular volume of lipids in the hexagonal lattice of a bilayer: no kink at T; kink at $T + \delta T$ without expansion of the environment; kink at the new equilibrium state at $T + \delta T$. The dot in the hexagonal face represents the position of the vertical glycerol backbone. See text for the indicated quantities.

molecular anisotropy [1]. This possibility is disregarded, however, because to our knowledge there is no evidence for increasing order with rising temperature.

The local shrinkage is depicted in outline in Fig. 3. If we assume a hexagonal prism for the molecular volume with a cross-section of 48.3 Å^2 [23] and, further, a kink to cause a shortening of the lipid chain within the same cross-section by about 0.8 Å, we obtain a reaction volume for the kink formation of −38.6 Å^3/molecule or ΔV_k = −23.2 cm^3/mol. From the amplitude of ultrasonic absorption of dipalmitoyl phosphatidylcholine vesicles, Gamble and Schimmel [7] calculated $|\Delta V|$ = 24.6 ± 3.0 cm^3/mol, in good agreement with our result.

However, the fact that the ultrasonic absorption amplitude yields $(\Delta V)^2$ and thus only the absolute value of ΔV led those authors to a misinterpretation of the reaction volume, although they recognized the true nature of the observed process. The overall reaction volume, namely, happens to be of almost the same absolute value, but of opposite sign: ΔV_{tot} = +24.2 cm^3/mol [28,29]. Regarding our sequential model we have to assume:

$$\Delta V_{tot} = \Delta V_k + \sum \Delta V_i \qquad (2)$$

where ΔV_k equals the reaction volume for the formation of a kink as measured by means of the ultrasonic absorption amplitude. For the as yet unspecified slower processes which were discussed above, we obtain with the given ΔV_{tot} and ΔV_k values a volume change of $\Sigma \Delta V_i$ = +48.8 cm^3/mol. In agreement with the density changes during the progress of the phase transition this expansion by 48.8 cm^3/mol means a strong decrease in turbidity. We indeed detected this after a pressure jump from elevated pressure to 1 atm (which corresponds to an upwards temperature jump) [4].

From our laser temperature-jump experiments we find no dependence of the

relaxation time τ on temperature. An Arrhenius-type dependence with a very small activation energy ought to be hidden in the experimental uncertainty. Since we do not observe a clear maximum of τ in the midpoint of the transition, we assume that the observed process is an isomerization independent of molecular interactions within the membrane lattice. We can thus write for the relaxation rate:

$$\frac{1}{\tau} = k_{12} + k_{21} \tag{3}$$

For the midpoint of transition the equilibrium constant:

$$K = \frac{k_{12}}{k_{21}} \tag{4}$$

must be unity and therefore $k_{12} = k_{21}$. Hence, the respective rate is $1/\tau_m = 2k_{12}$. With a relaxation time of 4 ns we obtain $k_{12}^{40^\circ C} = k_{21}^{40^\circ C} = 1.25 \cdot 10^8\ s^{-1}$. This agrees very well with the data of Gamble and Schimmel [7] from whose results $k_{12}^{40^\circ C} = k_{21}^{40^\circ C} = 5 \cdot 10^7\ s^{-1}$ is calculated.

A comparison between the described nanosecond processes occurring in the hydrophobic tail region of the lipid bilayer and the kinetic processes in small or large chain molecules (alkanes, vinylic polymers) reveals that the rate of rotation about C-C bonds does not depend strongly on the environment as long as hydrocarbon chains are considered in a hydrophobic environment. For polystyrene in various organic solvents, relaxation times between 10 and 50 ns were obtained from ultrasonic absorption [30,31]. Even a variation in solvent viscosity by a factor of 50 changes the relaxation time by a factor of only 3 [32]. Ultrasonic relaxation in higher liquid *n*-alkanes (n = 9—14) yielded relaxation times of 30—70 ns and showed that shorter chains rotate slightly faster than longer chains [33].

Also, we want to emphasize some parallels between the dynamic behaviour of liquid crystals and lipid bilayers. Jähnig [34] showed that for MBBA (*p*-methoxybenzylidene *p*-*n*-butylaniline) in the neighbourhood of the nematic-isotropic phase transition, the anisotropic attenuation of ultrasound is caused by two superposing effects: intramolecular *trans-gauche* rotations and the relaxation of the nematic order parameter. If the ultrasound frequency is chosen to be sufficiently small, these two contributions to the anisotropic sound attenuation are proportional to the respective relaxation times. The relaxation time for the C-C bond rotations, τ_i, exhibits and Arrhenius-type temperature dependence with an activation energy of 17.6 kJ/mol and the relaxation of the order parameter has a time constant τ_c with a critical increase in the neighbourhood of the phase transition. The rotational relaxation time τ_i was found to be 20 ns at 298 K.

The interpretation of the effects with a 'critical' relaxation behaviour appears to be more complex for vesicles and liposomes than for liquid crystals because two effects instead of only one are observed [3,4]. However, the similarity between the observations on the nanosecond effects supports the concept of a close relationship for the fast C-C bond rotations in both systems.

Finally, we shall discuss whether kinks in a lipid chain actually migrate with this chain. Diffusion coefficients (D) were obtained mainly by NMR and ESR

and yielded values of about $D = 10^{-8}$ cm^2/s in the fluid state of the membrane [35]. The expression:

$$D = \frac{\langle \lambda^2 \rangle}{4\tau} \tag{5}$$

derived for a two-dimensional random walk, where $<\lambda^2>$ is the mean of the square of the jump distance or the distance between two bilayer lattice points and τ the mean time lag between two jumps of a molecule from one lattice point to another, may serve for a rough comparison of time constants. The time lag obtained from the mentioned diffusion coefficients is then 190 ns if λ is assumed to be 8.7 Å. This time constant can be considered as a lower limit, since it was calculated for the fluid state. The result of this present work is that kink formation requires less than 10 ns. This means that lipid molecules assume and lose kinks while they reside at any chosen lattice point. Different energetic states such as chains with kinks and chains without kinks are therefore forwarded through the lattice preferentially by energetic transitions which are transmitted from one molecule to another. The hypothesis of a migration of individual molecules carrying a fixed energetic state can now be excluded. The translation of energetic states ought to be considered as a cooperative process and the growth and shrinkage of clusters of states ought to be seen in this light.

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