Biochimica et Biophysica Acta, 730 (1983) 239–244
Elsevier Biomedical Press

BBA 71614

EFFECTS OF PRESSURE AND PENTANOL ON THE PHASE TRANSITION IN THE MEMBRANE OF *ACHOLEPLASMA LAIDLAWII* B

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(Received August 16th, 1982)
(Revised manuscript received January 25th, 1983)

Key words: Membrane; Phase transition; Pressure effect; Pentanol; Benzyl alcohol; Fluorescence polarization; Differential thermal analysis; (A. laidlawii)

The phase transition of a complex, biological membrane was studied in relation to two variables: high pressure and the alcohols, pentanol and benzyl alcohol, in order to determine whether earlier studies of defined phospholipid bilayers may be applied to natural membranes. The isolated membrane of *Acholeplasma laidlawii* was used and three independent methods were chosen to detect the transition; optical transmission (giving T_t, an index of the end-of-melting temperature); fluorescence polarisation (giving T_p, the temperature midway through the change in polarisation which characterises the transition) and differential thermal analysis giving a record of the temperature range occupied by the endothermic process. Pressure increased T_t by 0.017 $K \cdot atm^{-1}$ and T_p by 0.016 $K \cdot atm^{-1}$, consistent with $dT/dP = T \cdot \Delta V/\Delta H$. Pentanol (and benzyl alcohol) lowered T_t, T_p and the temperature of the endotherm seen with differential thermal analysis. Thus the membrane transition responds to pressure and alcohols in agreement with thermodynamic theory.

Introduction

The use of high hydrostatic pressure to study the molecular structure and thermodynamic properties of cell organelles is well established [1–3] and in recent years has been extended to membranes [4]. The pure phospholipid liposome bilayer becomes more ordered at high pressure, undergoing an endothermic gel/liquid-crystalline transition at increased temperatures in accordance with the Clausius-Clapeyron relationship $dT/dP = T \cdot \Delta V/\Delta H$. Over the pressure range of several hundred atmospheres ($1\ atm = 1.01 \cdot 10^5\ N \cdot m^2$) ΔH and ΔV are both positive and constant [5,6], hence dT/dP is constant [7], and typically within the range 0.017–0.024 $K \cdot atm^{-1}$, depending on lipid composition. The main purpose of the experiments reported here was to establish whether cell membrane bilayers exhibit the same property, or whether lipid heterogeneity, membrane proteins or other features of natural membranes introduce complicating factors.

The membrane of *Acholeplasma laidlawii* B was chosen because its bilayer undergoes a well defined phase transition over a temperature range of about 25°C [8,9], the mid-point of which is largely determined by the composition of the fatty acids in the bilayer lipids [9]. In one study ΔH equalled $90 \pm 10\%$ of the value determined in bilayers made from extracted lipids (3.6 $cal \cdot g^{-1}$ and 3.9 $cal \cdot g^{-1}$ lipid, respectively) [8]. In another, cholesterol-free membranes enriched with elaidic acid gave $\Delta H = 11.3 \pm 2.4$ $cal \cdot g^{-1}$ lipid [10]. Spectroscopic measurements indicate that membrane proteins moderate the fluidity of the bilayer [11,12].

The volume expansion, ΔV, which occurs during the transition in *Acholeplasma* membranes is 2.1% of the membrane volume, i.e. approximately

4% of the lipid bilayer [13], as compared to 3.5% measured in phospholipid liposomes [4]. Other volume changes, attributable to membrane proteins, are also detected above the membrane transition temperature. However, using the second value for ΔH and the sole value for ΔV, the Clausius-Clapeyron equation predicts $dT/dP = 0.02\ K \cdot atm^{-1}$, similar to the value for phospholipid bilayers [4]. Clearly, it is of interest to measure dT/dP directly in *Acholeplasma* membranes, and to relate it to ΔH and ΔV.

A second purpose of these experiments was to measure the effect of alcohols on the transition temperature in the natural membrane. The transition temperature in phospholipid liposomes is reduced by the presence of alien molecules, approximated by $\Delta T = C_m \cdot R \cdot \Delta T^2/\Delta H$ [14].

Methods

Acholeplasma membranes were routinely prepared from cells grown without fatty acid supplement at 37°C, and were stored at −70°C [15]. The phase transition was detected by three methods; an optical transmission (i.e. turbidimetric) method [16,17], fluorescence polarisation and differential thermal analysis. In the first method, membranes, or liposomes prepared by vortex-mixing chloroform-methanol extracts of membranes, were suspended at an absorbance (450 nm) of 0.3 at room temperature in unbuffered water of pH 5–6, or in sodium acetate and acetic acid (pH 3.5, 5.5) or sodium hydrogen phosphate and potassium dihydrogen phosphate (pH 5.3, 7.7).

Transmission determinations of the phase transition were carried out at 546 nm using an Eppendorf photometer fitted with a triple cuvette (3 ml) heating block (atmospheric pressure) or with a stainless steel pressure cuvette fitted with sapphire windows [19]. In both cases the unstirred suspension was heated at $0.3\ K \cdot min^{-1}$ and its temperature was measured continuously to ±0.2 K with a thermistor. A frozen batch of membranes was thawed, resuspended in a selected solution and de-gassed in a vacuum immediately before determining its transition temperature. For measurements at high pressure the suspension was divided into two, one sample was scanned in the pressure vessel at atmospheric pressure and the other at high pressure. For measurements involving the addition of alcohol, the suspension was divided into three for simultaneous scanning. In all cases each sample was used once only.

For the measurement of fluorescence polarisation the buffer (pH 4.5 and 7.5) was a mixture of 0.133 M citric acid and 0.266 M disodium hydrogen phosphate. The membrane preparation was added to 2.6 ml buffered medium to give an absorbance (450 nm) of 0.1 at room temperature. 2 μl of a solution of 2 mM 1,6-diphenyl-1,3,5-hexatriene (DPH, Aldrich 'puriss' grade) in tetrahydrofuran was added with vigorous stirring, and the solution was incubated at room temperature for a further 15–20 min. The pH of the phosphate and acetate buffers is reduced no more than 0.2 at the highest pressure used [18].

Fluorescence polarisation determinations were performed on a T-format apparatus (Applied Photophysics, London) similar to the one described by Weber [20]. The excitation beam was polarised and the emission was analysed with Zeiss film polarisers. Monochromatic excitation light (360 nm) was filtered by a Corning CS7-54 broad band-pass filter and the emission observed through Corning CS3-75 sharpcut filters. A Julabo thermostatted water bath was used to control the cuvette temperature (±0.1 K) and the temperature of the sample was monitored directly with a precision thermistor (±0.1 K). Scattered light typically comprised only 1–2% of the total emission even at low temperature when the membrane suspension was particularly refractile. Corrections for this effect were not made.

The pressure vessel used for the fluorescence polarisation measurements comprised a block of stainless steel 10 $cm^2 \times 7$ cm deep, penetrated by holes in the form of a cross. Each hole was sealed by a quartz window (10 mm diameter, Spectrosil A, Optical Works, Ealing, London). The membrane suspension was injected through a small port into the cross-shaped space between the windows (total volume approx. 3 ml) which was subsequently pressurised via a flexible steel capillary tube connected to the injection port. The temperature of the vessel interior was monitored to ±0.1 K by a thermistor penetrating the steel block to within 1.0 cm of the interior space. The effect of hydrostatic pressure on the birefringency

of the quartz windows [21] was determined by a solution of fluorescein in glycerol at −6°C. Polarisation was affected over the pressure range 1–700 atm by only ±0.003 which is only slightly larger than the precision of the polarisation measurements. Corrections for this effect were not applied.

Fluorescence polarisation measurements were made at atmospheric pressure on up to three membrane suspensions, simultaneously heated in 2°C steps at 15-min intervals from approx. 10°C to 60°C. The polarisation of each sample was determined in rotation after the temperature was fully stabilised. Measurements using the pressure vessel involved a similar step-wise heating of a single sample, whose polarisation was determined at a nominal low pressure (to seal the windows) and at a selected higher pressure. The high pressure was applied and released after each heating step. In all cases each sample of membranes was used only once.

Differential thermal analysis was carried out using a Stanton-Redcroft analyser placed in a constant temperature cabinet at 24 ± 1°C. Water at 0°C circulated through the analyser. Typically membrane samples were about 10% dry weight in either water or buffer solution and were scanned in open pans at 4 K · min $^{-1}$ against a reference sample of water. Each sample served as its own control being heated through its transition two or three times before being treated with alcohol.

Results

Pressure

The onset temperature (T_t) of maximum optical transmission corresponds to the end-of-melting in the phase transition [16]. Fig. 1 shows a typical result obtained with membranes at atmospheric pressure and at 900 atm. High pressure increased T_t with either a reduction of, or no effect on, the amplitude of the transmission change. Fig. 2 summarises the data for unbuffered membranes, from which $dT/dP = 0.017$ K · atm^{-1}, and for buffered membranes whose dT/dP was not significantly different. One experiment with liposomes showed similar results.

Fluorescence polarisation at high pressure is shown in Fig. 3A. The graphs at each pressure

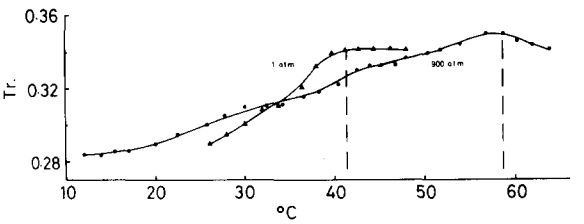

Fig. 1. Changes in the optical transmission of suspensions of *Acholeplasma laidlawii* B membrane at high and at atmospheric pressure. Typical results are shown for membranes in unbuffered water, pH 5.4. The dashed lines indicate T_t, the onset temperature of maximum transmission (plotted in arbitrary units), and corresponds to the end-of-melting in the phase transition (see text).

were typically triphasic, although the change of slope at low and high temperature was gradual. The decrease in polarisation, which is characteristic of the melting process of the phase transition, occurred at higher temperature under pressure. The effect of temperature on polarisation either

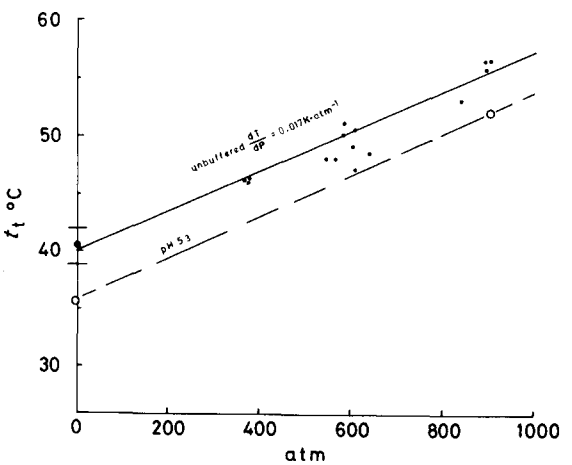

Fig. 2. The effect of pressure on the phase transition temperature of *Acholeplasma laidlawii* membranes, determined by the onset of maximal optical transmission, T_t. Data are shown for experiments on separate batches of membranes in unbuffered aqueous suspensions at high pressure. The one atmosphere control points are plotted as a mean ± S.D. ($n = 16$) for convenience. A linear regression line is also drawn for the data from membranes suspended in sodium acetate/acetic acid buffer at pH 5.3. Three measurements were made at atmospheric pressure and three at 900 atm (not plotted), $r = 0.83$. The points (O) were obtained from one experiment with unbuffered liposomes, pH 4.5, and fortuitously lie on the regression line for membranes at pH 5.3.

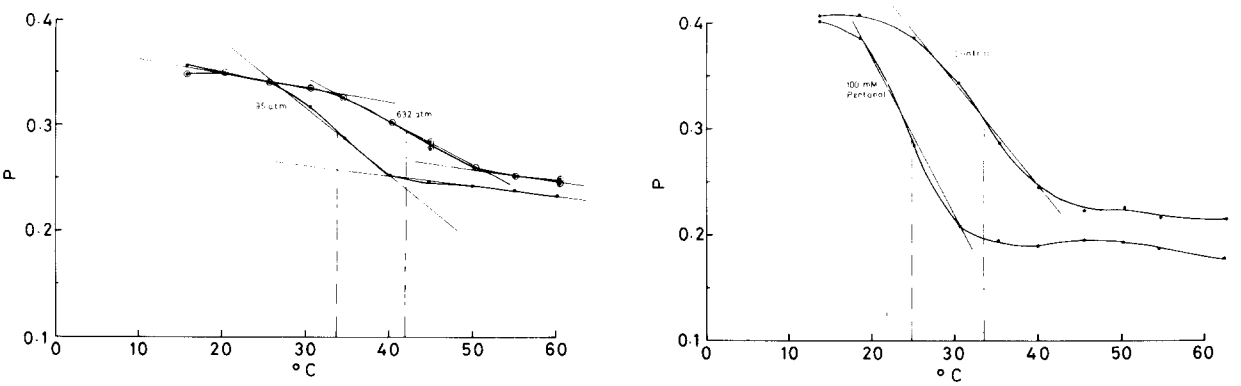

Fig. 3. The phase transition of *Acholeplasma laidlawii* membranes, subjected to pressure (A), and to pentanol (B), determined by fluorescence polarisation. Typical results are shown. (A) Pressure: A membrane suspension (pH 4.5) was subjected to 95 atm and 632 atm alternately whilst being heated in the fluorescence polarisation pressure vessel (see text). (B) Pentanol: A membrane suspension (pH 7.5) was divided into dosed and control samples and polarisation values were determined over the 10–60°C range.

side of the transition was very small and was not greatly affected by pressure. A convenient estimate of the effect of variables such as pressure on the transition as detected by fluorescence polarisation is obtained by measuring the temperature at which the curves in Fig. 3 are maximally separated over the steep section giving ΔT_{pmax}. Alternatively, the difference in temperatures at which the curves are mid way through their change in polarisation (ΔT_p) may be obtained from straight lines fitted to the curve by inspection. Table I summarises the data obtained in this way and it is clear that the estimates for dT_p/dP (0.016 $K \cdot atm^{-1}$) and dT_{pmax}/dP (0.017 $K \cdot atm^{-1}$) agree with the corresponding value obtained from the optical transmission method (0.017 $K \cdot atm^{-1}$).

Pentanol

Pentan-1-ol and benzyl alcohol reduced T_t in a dose-dependent manner. The values for T_t in unbuffered suspension, pH 5.5, containing the given concentrations of pentanol were: 41.4 ± 1.2°C (pentanol-free control); 36.2 ± 1.2°C (50 mM); 32.2 ± 1°C (100 mM); 26.5 ± 2.2°C (200 mM) (n = 4). Similarly benzyl alcohol reduced T_t from a control value of 42.8°C to 34.0°C 120 mM) (n = 6). T_t is affected by large changes in pH both in the presence and absence of alcohols (Macdonald and

TABLE I

EFFECT OF PRESSURE ON THE PHASE TRANSITION OF ACHOLEPLASMA LAIDLAWII B MEMBRANES DETERMINED BY FLUORESCENCE POLARISATION

Data on each line were obtained from sub-samples of the same batch of membranes at pH 4.5.

Expt. No.	T_p, temperature midway through change in polarisation at given pressure (°C)		dT_p/dP ($K \cdot atm^{-1}$)	ΔT_{pmax}, maximum temperature difference between polarisation-temperature curves (K)	dT_{max}/dP ($K \cdot atm^{-1}$)
	Low pressure	High pressure			
1	38.5 (163 atm)	46.8 (619 atm)	0.0182	9.0	0.0197
2	36.2 (95 atm)	44.2 (612 atm)	0.0155	7.5	0.0145
3	33.7 (95 atm)	42.0 (632 atm)	0.0154	10.0	0.0186
Mean ±S.D.			0.016 ±0.001		0.017 ±0.002

Cossins, in preparation). The effect of pentanol on the amplitude of the change in optical transmission was variable; in some cases the transmission curves appeared to be simply displaced down the temperature axis and in others the transmission-temperature curve was very steep, peaking at a relatively low temperature.

The effect of pentanol on fluorescence polarisation was readily detectable. 100 mM at pH 7.5 lowered T_p by 8.4 ± 0.53°C, ($n = 3$) (Fig. 3B).

Differential thermal analysis of concentrated membrane suspensions revealed characteristic endotherms whose peak shifted approximately 10°C to lower temperatures in the presence of pentanol at concentrations in the 300–800 mM range. No attempt was made to quantify the reduction in transition temperature more precisely in view of the low accuracy inherent in this method.

Discussion

T_t marks the end-of-melting in *Acholeplasma* membranes and phospholipid bilayers [16,17]. Fluorescence polarisation is a measure of the motional characteristics of membranes. Time resolved anisotropy measurements have indicated that the anisotropy or polarisation measured under steady-state illumination is dictated mainly by the degree of orientational constraint offered by the highly anisotropic membrane interior, and is largely a measure of membrane order [22].

Pressure increases T_t, T_p and T_{pmax} by 0.017, 0.016 and 0.017 $K \cdot atm^{-1}$ respectively, broadly consistent with the 0.02 $K \cdot atm^{-1}$ predicted in the Introduction. These data are similar to those obtained from liposomes made from mixed lipids extracted from *Azotobacter* (0.018 $K \cdot atm^{-1}$ [23]) and from lipids extracted from *Acholeplasma* membranes (this paper). It is interesting that in rabbit macrophage membranes spin-labelled with fatty acids, a characteristic temperature-dependent change in the rotational correlation time shifted from 2.5°C to 10°C on application of 270 atm [24]. This gives a rather high dT/dP of 0.027 $K \cdot atm^{-1}$. A recently published study of pyrene excimer fluorescence in human erythrocyte membranes under pressure showed a shift in a thermotropic transition of 0.02–0.022 $K \cdot atm^{-1}$ [25].

The application of the Clausius-Clapeyron equation to such a weakly cooperative (i.e. broad) transition as occurs in *Acholeplasma* membranes is open to doubt. However, the results reported here are sufficiently close to those obtained with highly cooperative transitions in pure lipid liposomes [4] where the equation does apply, to suggest that the equation can also apply to complex membrane transitions.

Turbidimetric, fluorescence and differential thermal analysis methods all showed that pentanol caused a shift in the membrane transition to lower temperatures. Benzyl alcohol has an effect on T_t similar to that of pentanol, consistent with their similar oil : water coefficients. T_t was reduced 9.2 K in the presence of 100 mM pentanol, unbuffered pH 5.5, whilst T_p was lowered similarly (8.4°C at pH 7.5).

Generally the depression of the *Acholeplasma* membrane transition temperature by alcohols is the same as that seen in pure phospholipid liposomes and it seems reasonable to invoke the same mechanism [14].

The conclusion from this work is that the phase transition in *Acholeplasma* membranes responds to hydrostatic pressure and alcohols in a way that is consistent with thermodynamic theory. However, the Arrhenius break temperature for the membrane-bound ATPase shows a different response. Although it is increased by 0.015 $K \cdot atm^{-1}$ [15] compared to 0.017 $K \cdot atm^{-1}$ for the membrane transition reported here, unlike the membrane transition, it is not lowered by pentanol [15].

Acknowledgement

We thank Alison Stewart for skilled technical assistance.

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