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PHASE TRANSITIONS OF PHOSPHOLIPID BILAYERS FROM AN UNSATURATED FATTY ACID AUXOTROPH OF *ESCHERICHIA COLI*

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Summary

Total phospholipids were extracted from cells of temperature sensitive unsaturated fatty acid auxotrophs of *Escherichia coli* (K-12 UFA^{ts}) grown at 28°C (PL28), and at 42°C in the presence of 2% KCl as an osmotic stabilizer (PL42 (KCl)). From the analysis of fatty acids, it was shown that the content of unsaturated fatty acids of PL42 (KCl) is only 9% of the total fatty acids, while that of PL28 is 54%. The thermal phase transitions of the bilayers prepared from the phospholipid fractions were studied by proton magnetic resonance. The line widths of the methylene signals and the sums of the methylene and methyl signal intensities were plotted against reciprocal values of absolute temperature $1/T$ or temperature itself. From the plots phase transitions were detected at about 19°C for PL28 and at 43°C for PL42 (KCl). In spite of its complex composition of fatty acids a highly cooperative transition was observed in the case of PL42 (KCl). It was also suggested that the phospholipids bilayers in the biomembranes of this strain at the growth temperature (42°C) are in the state where the gel and liquid crystalline phases coexist.

Introduction

The phase transitions of the bilayers of phospholipids extracted from *Escherichia coli* membranes or the membrane itself have been investigated by several methods [1—9]. In earlier works unsaturated fatty acid auxotrophs of *E. coli* supplemented with various unsaturated fatty acids have been frequently used, because fatty acid compositions in their membranes are simpler and can be altered in a limited range by a variety of supplements of fatty acids. As pointed out by Akamatsu [10] and Broekman and Steenbakkers [11], *E. coli* K-12

UFA^{ts}, one of the unsaturated fatty acid auxotrophs, can grow at 42°C under osmotically stabilized conditions without any unsaturated fatty acid. The membranes have an abnormally low content of unsaturated fatty acids in phospholipids, and reveal fragile character.

In the present study we examined the properties of the phospholipid bilayers prepared from the auxotrophs by the use of proton magnetic resonance. Although a nuclear magnetic resonance (NMR) technique is widely used in the study of phase transitions of synthetic phospholipid-water systems, it has not been employed frequently for the study on phospholipids of *E. coli*. One of the reasons for this is that the complexity of the system makes it difficult to give an explanation for the change in their NMR spectra. However, we found a remarkable difference in the behavior on the phase transition between the normal cells grown at 28°C and those grown at 42°C under osmotically stabilized conditions, and show the usefulness of NMR in the study of such complex systems as the phospholipid bilayers from *E. coli*.

Materials and Methods

A temperature-sensitive auxotroph of *E. coli* K-12 UFA^{ts} requiring unsaturated fatty acids was grown by the use of a jar fermenter (200 l) in aerated synthetic minimum medium [12] containing 1% casamino acids (Difco, vitamin free) and 0.25% glycerol at 28°C with 2% KCl as an osmotic stabilizer at 42°C without supply of unsaturated fatty acids. Cells cultured overnight were harvested. The total phospholipids were extracted from cells with chloroform/methanol according to the method of Bligh and Dyer [13], and further purified by silicic acid column chromatography. Phospholipid fractions were eluted with a chloroform/methanol (v/v, 3 : 2) mixture. Phospholipid-containing fractions were evaporated to dryness on a rotary evaporator, dissolved in chloroform and lyophilized. All samples used in the present experiment were prepared from the same lot.

The total phospholipids were separated by silica gel thin-layer chromatography. The thin-layer plates were developed with chloroform/methanol/water (v/v, 65 : 25 : 4) and the spots were detected by exposure to iodine vapor. The mole fractions of the separated phospholipids were determined from the phosphorus assay of the spot according to the method of Bartlett [14].

Fatty acid methyl esters were obtained by transesterification with HCl/methanol, and the methyl esters were determined by gas-liquid chromatography on a column (0.3 × 300 cm) packed with 15% diethylene glycol succinate on chromosorb W, using methyl palmitate as internal standard. A Shimadzu GC-4APT gas chromatograph equipped with an integrator, model ITG-2A, was used as the analyzer.

Lipid samples in ^2H_2O (p^2H was adjusted by the addition of NaO^2H or ^2HCl) were sonicated for 5 min at 5°C in nitrogen atmosphere at 20 kHz with a Branson sonifier model B-12. The p^2H values of the sonicated dispersions were given by reading a Hitachi-Horiba pH-meter model M-5 equipped with a microelectrode (Ingold Electrodes Inc.).

The proton magnetic resonance spectra of the dispersions were obtained at 100 MHz with a JEOL PFT-100 pulse Fourier transform NMR system locked

on deuterium and equipped with temperature control apparatus. Sample concentration in the NMR measurements was 10 mg phospholipids per 0.4 ml deuterium oxide. Repetition rate was 5 s and the spectra were accumulated 20 or 100 times in the time domain.

Electron microscopic measurements were performed with a Hitachi HU-11B electron microscope at 75 kV. Samples were stained with a 2% phosphotungstic acid solution.

Results

Compositions of phospholipids and fatty acids in E. coli K-12 UFA^{ts} membrane lipids

The compositions of phospholipids from *E. coli* K-12 UFA^{ts} grown at 28°C (PL28) and those grown under the protected conditions at 42°C (PL42 (KCl)) are summarized in Table I. Both PL28 and PL42 (KCl) contain about 80% phosphatidylethanolamine as a main phospholipid component. On the other hand, they differ from one another in the fractions of cardiolipin and phosphatidylglycerol, suggesting that PL42 (KCl) possesses a higher negative charge density than PL28 since the former contains much more cardiolipin which has two phosphate groups.

The total fatty acid compositions of phospholipids in PL28 and PL42 (KCl) are summarized in Table II. PL28 contains 54% unsaturated fatty acids, while PL42 (KCl) has very low content (only 9%) unsaturated fatty acids. Furthermore, the 36% myristic acid content in PL42 (KCl) was unusually high in contrast with 0.5—5% in the normal composition of phospholipids from *E. coli.* The fatty acid compositions of the individual phospholipids of PL42 (KCl) are summarized in Table III. From the data in the table, it is seen that the content of unsaturated fatty acids is comparable among three kinds of phospholipids.

Electron microscopy

Electron microscopic photographs of PL28 and PL42 (KCl) dispersions by negative staining are shown in Figs. 1A and B. In the case of PL28, the diameters of the vesicles distribute approx. 200—400 Å and the averaged value is about 250 Å. On the other hand PL42 (KCl) dispersion is more liable to aggregate through the sample preparation for an electron microscopic measurement and the sizes of the vesicles distribute in a wider range (from 200 to 1200 Å) as shown in Fig. 1B. The results reveal that both PL28 and PL42 (KCl) dispersions used in the present experiment are single bilayer vesicles.

TABLE I

THE COMPOSITION OF PHOSPHOLIPIDS IN PL28 AND PL42 (KCl)

Phospholipid	PL 28 (mol %)	PL 42 (KCl) (mol %)
Phosphatidylethanolamine	78.3	79.8
Cardiolipin	4.1	15.7
Phosphatidylglycerol	11.3	4.5
Lysophosphatidylethanolamine	6.2	—

TABLE II

THE FATTY ACID COMPOSITIONS OF TOTAL PHOSPHOLIPIDS IN PL28 AND PL42 (KCl)

Abbreviations: 12 : 0, lauric acid; 14 : 0, myristic acid; 14 : 1, myristoleic acid; 16 : 0, palmitic acid; 16 : 1, palmitoleic acid; 18 : 1, *cis*-vaccenic acid; 17Δ and 19Δ, cyclopropane ring-containing fatty acids with the indicated carbon number.

Fatty acid	PL 28 (mol%)	PL 42 (KCl) (mol%)
12 : 0	1.26	1.50
14 : 0	2.86	36.17
14 : 1	1.56	3.73
16 : 0	41.27	53.45
16 : 1 + 17 Δ	31.68	3.11
18 : 1 + 19 Δ	21.39	2.04
Total unsaturated fatty acids *	54.63	8.88

* Including cyclopropane fatty acids.

TABLE III

FATTY ACID COMPOSITIONS OF EACH SEPARATED PHOSPHOLIPID FRACTION OF PL42 (KCl)

Fatty acid	Phosphatidylethanolamine (mol%)	Cardiolipin (mol%)	Phosphatidylglycerol (mol%)
12 : 0	1.62	2.61	1.30
14 : 0	39.65	36.73	32.79
14 : 1	1.71	3.90	2.14
16 : 0	50.71	52.88	55.56
16 : 1 + 17 Δ	3.25	2.12	5.61
18 : 1 + 19 Δ	3.06	1.76	2.60
Total unsaturated fatty acids *	8.02	7.78	10.35

* Including cyclopropane fatty acids.

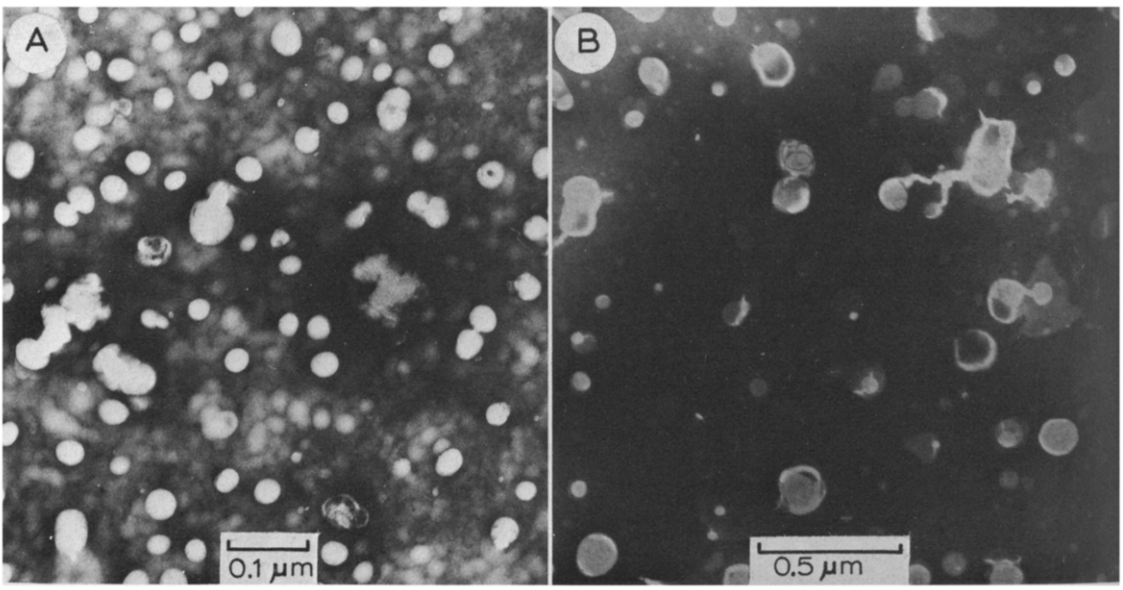


Fig. 1. Electron microscopic photographs of PL28 vesicles (A) and PL42 (KCl) vesicles (B). Samples were stained with a 2% phosphotungstic acid solution.

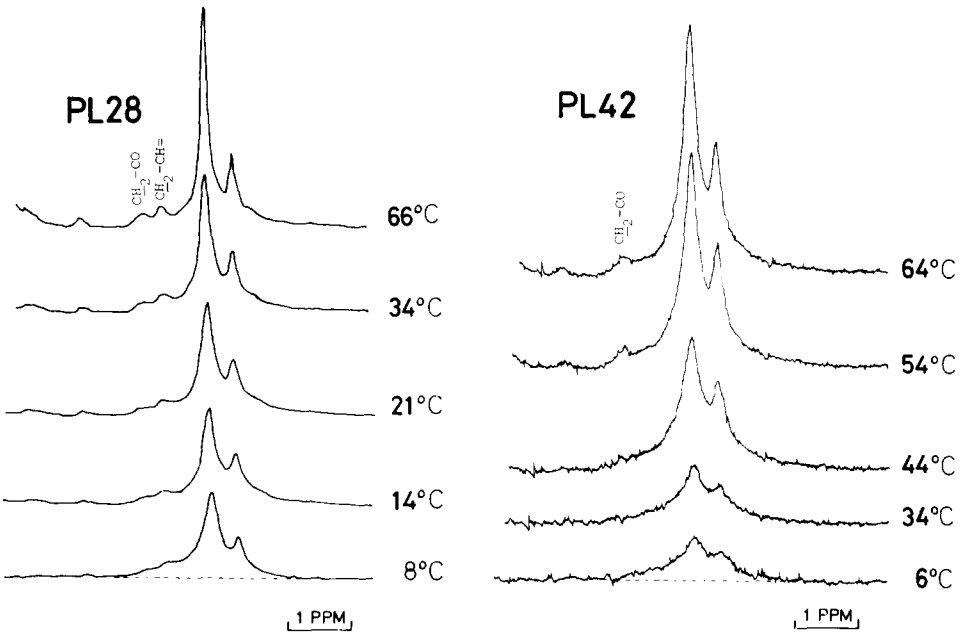


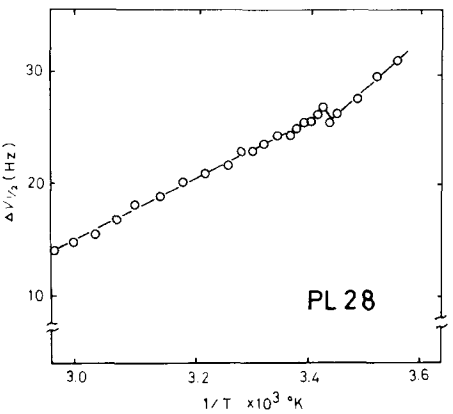
Fig. 2. 100 MHz Proton NMR spectra of PL28 (left) and PL42 (KCl) (right) vesicles in deuterium oxide at various temperatures. Broken lines are baselines used for the determination of linewidths and intensities as mentioned in the text.

Effect of temperature on the NMR spectra of E. coli PL28 and PL42 (KCl) vesicles

Fig. 2 shows the 100 MHz NMR spectra of the dispersions of PL28 and PL42 (KCl) at various temperatures. The two strong signals at higher and lower field in Fig. 2 are assigned to the methyl and methylene protons of hydrocarbon chains, respectively. The assignments of the other signals followed those of Chapman and Morrison [15]. The experiments of temperature dependence were performed only by raising the temperature. Linewidths and intensities were determined as follows. Base lines were assumed to be the straight lines indicated in Fig. 2. The linewidth of the methylene signal was determined as the width at half peak-height ($\Delta\nu_{1/2}$). For PL42 (KCl) at lower temperatures the linewidth could not be determined directly because of the overlap of unresolved signals. In that case twice the left-half width of the methylene signal at half peak-height was considered as the linewidth of this signal. The total intensity of the methyl and methylene signals were determined by measuring the area above the base line.

The effect of temperature on the linewidth of the methylene signal of PL28 vesicles is shown in Fig. 3 where the linewidth are plotted against $1/T$. The plot was biphasic and could be resolved into two linear lines. The region around the reflection point may correspond to the phase transition from the gel to the liquid-crystalline state as will be discussed later.

The intensity changes of the methylene and methyl signals of PL28 and PL42 (KCl) against temperatures are given in Fig. 4. A small change is observed around 19°C in the plot of PL28, which corresponds to the reflection point in



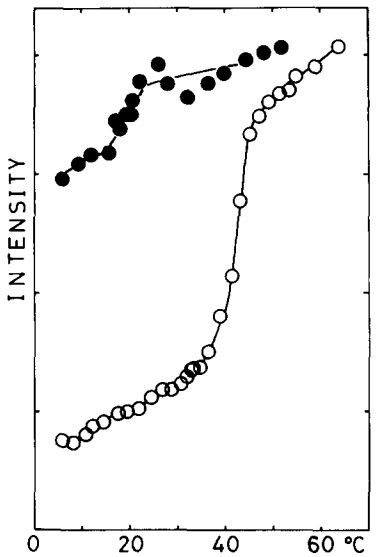


Fig. 3. A temperature dependence of the linewidth of the methylene signal of PL28 vesicles at p^2H 6.7.

Fig. 4. Plots of the sum of the intensities of the methyl and methylene signals of PL28 vesicles at p^2H 6.7 and PL42 (KCl) at p^2H 5.3 against temperature. ●, PL28, ○, PL42 (KCl).

Fig. 3. This fact also indicates that the phase transition of PL28 vesicles takes place in this region. On the other hand for the plot of PL42 (KCl) a fairly large change appeared at a higher temperature, the midpoint of which is read as 42.5°C.

Fig. 5 shows the plots of the linewidth and peak height of the methylene signal of PL42 (KCl) vesicles against $1/T$. The change in the peak height is very

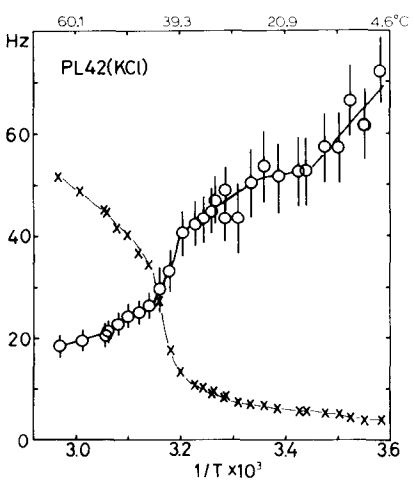


Fig. 5. Temperature dependence of the linewidth and the peak height of the methylene signal of PL42 (KCl) vesicles at p^2H 5.3. ○, linewidth; X, peak height.

large. The midpoint of the change is 43°C, which is in good agreement with that read from intensity change in Fig. 4. The linewidth of the methylene signal also changes abruptly in this region. These facts indicate that the phase transition from the gel to the liquid-crystalline states in the PL42 (KCl) vesicles occurred at about 43°C and is highly cooperative. The much higher transition temperature of PL42 (KCl) compared to that of PL28 can be attributed to the large amount of saturated fatty acids.

Fig. 4 shows that most of the methylene signals of PL42 (KCl) are broadened and can not be detected in the high resolution NMR spectrum at the gel state because of a highly restricted motion of hydrocarbon chains, while most of the methylene signals of PL28 can be detected even at the gel state. This fact suggests that the packing of the hydrocarbon chains in the PL28 vesicles at the gel state is much looser than those in the PL42 (KCl) vesicles and their motions are not so restricted resulting from weak lipid-lipid interaction.

p^2H dependence of the transition temperatures

In order to evaluate the effect of the polar groups of phospholipids on the transition temperature, p^2H dependence was examined in the region of p^2H 4—10 for PL28 and PL42 (KCl) vesicles. The p^2H dependence of the transition temperature of PL28 vesicles is not large but shows the highest transition temperarure in the vicinity of p^2H 6, which is close to the pH of the isoelectric point of phosphatidylethanolamine [16]. It indicates that the ionic force among the charged headgroups of phosphatidylethanolamine is an important factor for the stability of the gel state. p^2H dependence for PL42 (KCl) is also not so remarkable. These results coincide with those observed for another kind of fatty acid auxotroph by Overath et al. [2].

Discussion

The fatty acid compositions in Table II show that PL42 (KCl) possesses quite a small amount of unsaturated fatty acids and the value is the lowest among the fatty acid contents of *E. coli* phospholipids as far as examined. The discrepancy of fatty acid compositions of PL42 (KCl) between the previous [10] and present works may be due to the change in the growth condition, though main features are similar in both cases.

The content of cardiolipin of PL42 (KCl) is larger than that of PL28. The larger negative charge density due to cardiolipin may be necessary for the phospholipids of PL42 (KCl) to form a stable membrane structure. Phosphatidylethanolamine, the main component of the phospholipid fraction, has a very low affinity to water and it is hard to disperse in water by sonication. It is also known that the polar groups of phosphatidylethanolamine form net-like structures in a monolayer at air/water interface and the molecules are closely packed in it [17]. To form a stable bilayer structure of liposomes in water it may be necessary that negative charges distribute over all the surfaces of liposomes and perturb the formation of net-like structures of phosphatidylethanolamine. Acidic phospholipids such as cardiolipin and phosphatidylglycerol may play this role in *E. coli* membranes. Then three kinds of phospholipids, phosphatidylethanolamine, cardiolipin and phosphatidylglycerol are expected to distribute

homogeneously. Table III reveals that there is no particular deviation in fatty acid compositions of the phospholipids. The fact also supports the idea of homogeneous distribution of the lipid molecules in the membrane structure of the *E. coli* cells grown at 42°C with 2% KCl.

It is well known that linewidths of the methylene proton signals of hydrocarbon chains contain information on the intra- and intermolecular motion of phospholipids [18—20]. Kroon et al. [20] examined a temperature dependence of the linewidths of the methylene proton signals of lecithin bilayers in magnetic fields of different strengths. Their results suggest that the linewidth of a methylene signal at 100 MHz is more sensitive to the motional state of phospholipids than that at 220 MHz, because the linewidth due to the chemical shift difference in the several kinds of methylene protons of hydrocarbon chains becomes larger compared with the linewidth due to the molecular motion in the latter case. The methylene signal in Fig. 2 is an overlapped one with many kinds of methylene proton signals. We assume the signal, however, as a single singnal at the first approximation in the case of PL28 where most of the methylene protons give the signal even at the gel state.

There are three contributions to the linewidth of a NMR signal as follows,

$$\pi\Delta\nu_{1/2} = \frac{1}{T_2^*} = \frac{1}{T_{2e}} + \frac{1}{T_{2d}} + \frac{1}{T_{2i}} \tag{1}$$

where $\Delta\nu_{1/2}$ is the linewidth of the signal, T_2^* is the apparent transverse relaxation time, T_{2e} is the transverse relaxation time accompanied by the energy transition, T_{2d} is the transverse relaxation time without the energy transition, T_{2i} is the transverse relaxation time due to the magnetic field inhomogeneity. $1/T_{2e}$ and $1/T_{2d}$ depend on the molecular motion but $1/T_{2i}$ does not directly. Since the molecular motion is a function of temperature, it can be said that the temperature dependence of the signal width is mainly due to the terms of $1/T_{2e}$ and $1/T_{2d}$. Thus Fig. 3 suggests that the motional state of phospholipids in bilayers changes at about 19°C. This was attributed to the gel liquid-crystalline phase transition of the hydrocarbon chains. The difference in the slopes of the two intersecting lines in Fig. 3 shows that the apparent activation energy of the phospholipid motion is larger in the lower temperature range than in the higher temperature range. This is also compatible with the interpretation of the gel liquid-crystalline phase transition.

The temperature dependence of linewidths of PL42 (KCl) is more complicated because the number of methylene groups contributing to the apparent signal also changes with temperature as shown in Fig. 4. The temperature dependence of the intensity, however, clearly indicates highly cooperative phase transition around 43°C for PL42 (KCl) in spite of wide distribution of vesicle sizes and complexed fatty acid composition. Baldassare et al. [9] investigated the phase transition of the *E. coli* membranes containing large amounts of unsaturated fatty acids by the use of differential scanning calorimetry. They showed that the phase transition is sharp and highly cooperative, when *cis*-monoenoic acid (*cis*-Δ^{11}-18 : 1) occupies 90% of the total fatty acids, but the transition is broadened when the content of another *cis*-monoenoic acid (*cis*-Δ^{9}-16 : 1) becomes larger, in spite of high content (93%) of total unsaturated fatty acids. Our results suggest that complex composition of saturated fatty acids

does not prevent strong lipid-lipid interaction in contrast to the case of unsaturated fatty acids, especially when difference in hydrocarbon chain lengths is small.

It is interesting to note that the transition ends around 50°C in the case of PL42 (KCl). Thus the lipid bilayers in the membrane of the auxotroph of *E. coli* at the growth temperature are on the way of phase transition and the gel- and the liquid-crystalline phases may coexist separately in the membrane. In general a growth temperature is above the temperature of the gel liquid-crystalline transition as is shown for PL28. In the case of the auxotroph supplemented with elaidic acid, the growth temperature is just above the upper end of the phase transition [8]. The above evidence for PL42 (KCl), therefore, may be correlated with the low growth rate of the auxotroph, i.e., the phase separation may lower the functions of the membranes. The osmotic fragility, however, is not due to the phase separation, because Baldassare et al. [9] showed evidence that the membrane lipids from *E. coli* cultured without an osmotic stabilizer are also in the state of phase separation at the growth temperature (37°C).

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