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LIPOSOMAL MEMBRANES

XI. A SUGGESTION TO STRUCTURAL CHARACTERISTICS OF ACIDO-THERMOPHILIC BACTERIAL MEMBRANES

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To understand the role of ω-cyclohexyl fatty acid residue of lipids in acido-thermophilic bacterial membranes, three unusual phosphatidylcholines, 1,2-di-11-cyclohexylundecanoyl-L-α-phosphatidylcholine (11_{CY}PC), 1,2-di-13-cyclohexyltridecanoyl-L-α-phosphatidylcholine (13_{CY}PC), and 1-13-cyclohexyltridecanoyl-2-11-cyclohexylundecanoyl-L-α-phosphatidylcholine (1-13_{CY}-2-11_{CY}PC) were prepared and the steady-state fluorescence anisotropy of 1,6-diphenylhexatriene (DPH) in the hydrophobic domain of these liposomal bilayers was determined. Compared with the case of dipalmitoyl (DPPC) or dimyristoyl phosphatidylcholine (DMPC), introducing the ω-cyclohexyl moiety onto lecithins makes the bilayers fluid below the phase transition temperature, while immobilizes them above the phase transition temperatures. The properties of the unusual phosphatidylcholine liposomes suggested by the steady-state fluorescence anisotropy investigation were in good agreement with those obtained from the thermotropic and permeability investigations. Results obtained are discussed from the view point of the role and function of lipid membranes of acido-thermophilic bacteria which contain unusual fatty acids.

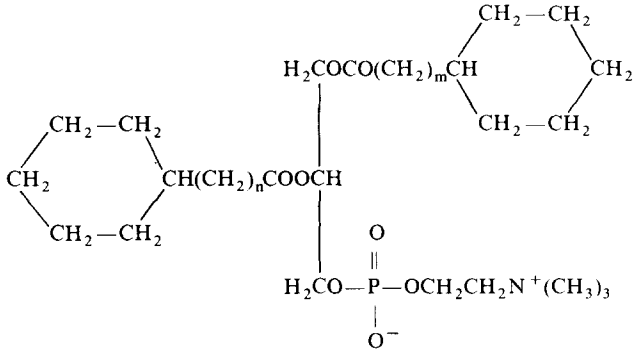
Introduction

Acido-thermophilic bacteria are characterized by their tolerance to high temperature (55–90°C) and acidity (pH 1–5). *Bacillus acidocaldarius*, one of these bacteria, has been isolated from the acidic hot springs of Yellow Stone and Hawaii Volcano National Park in U.S.A. by Darland and Brock [1], by Pisciarelli in Italy [2] and by Beppu and Tamagawa in Japan [3]. Oshima and Ariga [4] have found that ω-cyclohexyl undecanoic and tridecanoic acids are contained in ten strains of *B. acidocaldarius* isolated from different Japanese hot springs. These cyclohexyl moiety-containing fatty acids were found also in a thermophilic bacterium isolated fom Italian hot springs [2,5]. Very recently Oshima and coworkers [6] stated that a bacteriophage φNS11 also contains ω-cyclohexyl fatty acids [6]. These unusual fatty acids are involved as the esters in glyceride type lipids [2,4]. These facts suggest to us that the cytoplasmic membrane rather than the cell wall of the bacteria may contribute to thermostability and protection from an acidic environment. Especially, unusual fatty acid-containing lipids may play a special role to keep the pH gradient across the cell membranes even at elevated temperatures [7].

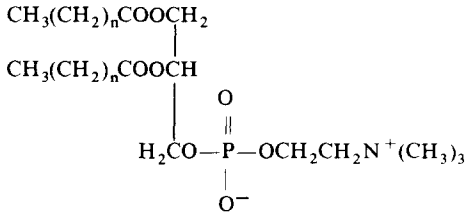
Now, we have to answer the question why biomembranes of these acido-thermophilic bacteria consist of unusual lipids bearing ω-cyclohexyl fatty

Abbreviations: PC, phosphatidylcholine; DPH, 1,6-diphenylhexatriene; DPPC, dipalmitoyl phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine.

acids. To understand the role of the cyclohexyl moiety in the function of the bacterial membranes, we have prepared the phosphatidylcholines carrying ω-cyclohexyl fatty acids (see Scheme 1) [34]



(1) $m=10, n=10$ $11_{CY}PC$ $T_c=13°C$
(2) $m=12, n=12$ $13_{CY}PC$ $T_c=33.5°C$
(3) $m=12, n=10$ $1\text{-}13_{CY}\text{-}2\text{-}11_{CY}PC$ $T_c=9°C$



(4) $n=12$ DMPC $T_c=23°C$
(5) $n=14$ DPPC $T_c=41°C$

Scheme 1. Structure of the phosphatidylcholines employed in this work.

and measured the steady-state fluorescence anisotropy of 1,6-diphenylhexatriene (DPH) in the hydrophobic domain of liposomal membranes compared with that of dipalmitoyl phosphatidylcholine (DPPC) and dimyristoyl phosphatidyl choline (DMPC) liposomes. The latter two phosphatidylcholines were selected from the view point of structural analogy in the length of the acyl residue as suggested by a CPK-molecular model, such as DPPC was to 13_{CY}PC, and DMPC to 11_{CY}PC, respectively. Although the chemical structure of major head group of bacterial lipids has not been fully elucidated yet, phosphatidylcholine with cyclohexyl acyl chains is a minor, but definite, component of the bacterium [34]. In addition, phosphatidylcholine is useful in obtaining information on the properties of glycerolipids, since these compounds form liposomes easily.

A growing number of papers have recently appeared, where the steady-state depolarization measurements have been utilized to estimate the microviscosity of the hydrocarbon domain of liposomal bilayers in which fluorophores have been embedded [8]. The viscosity reflected in depolarization measurements is that of the solvent in the immediate vincinity of the rotating unit, where the depolarization of a spherical rotor is described by Perrin's law [9] of isotropic depolarization. However, this is strictly valid only for simple exponential decay of the emission [10]. Recently, from a nanosecond time-resolved fluorescence anisotropy measurement of DPH in liposomal bilayers, it has been shown that the emission anisotropy curve is fitted to a sum of two component decays [11,12]. In addition, Jähnig [13] has proposed that the steady-state measurements of fluorescence anisotropy can provide the order parameter, S, in good approximation. Hence in this work we tried to estimate the order parameter of liposomal bilayers of unusual phosphatidylcholines from the steady-state fluorescence anisotropy of DPH embedded in different liposomes and we discuss it comparing results of DSC and permeability measurements.

Materials and Methods

Materials. Detailed procedures to synthesize and purify the phosphatidylcholines (1–3 in Scheme 1) are described elsewhere (Endo, T., et al., Ref. 34). Dipalmitoyl-DL-α-phosphatidylcholine (DPPL) and dimyristoyl-L-α-phosphatidylcholine (DMPC) were purchased from Sigma Chemical Co., St. Louis, MO. 1,6-Diphenylhexatriene (DPH) was obtained from Aldrich Chemical Co., Milwaukee, WI. Other organic and inorganic chemicals were commercially available at analytical grade and were used without further purification.

Preparation of liposomes. Liposomes containing DPH were formed and isolated by the same method as that described previously [14,15]. To prepare a thin film, a chloroform solution of phosphatidylcholines was mixed with DPH dissolved in

tetrahydrofurane. The molar ratio of DPH to phosphatidylcholine was maintained at 1 to about 130 [16] through all the runs, since it is well established that a ratio of phosphatidylcholine to DPH as low as 50 has no effect on the fluorescence depolarization results [17]. After evaporating solvents under reduced pressure using a rotary vacuum evaporator, the remaining thin film was dispersed in 0.10 M aqueous NaCl solution by shaking on a Vortex mixer with glass beads. Dispersing and swelling were carried out at a temperature that was about 10°C higher than the phase transition temperature of the phosphatidylcholines employed. The resulting suspension was subjected to ultrasonic irradiation under nitrogen atmosphere using a Tomy UR-200P probe-type sonifier at 25 W for 5 min at 1 min intervals. Gel-filtration was carried out on a Sephadex G-50 column (0.8 × 28 cm) equilibrated in 100 mM aqueous sodium chloride, by which an almost transparent suspension of small unilamellar liposomes was obtained. The concentration of liposomes was determined as inorganic phosphate as described by Allen [18].

For the steady-state fluorescence anisotropy measurements, the resulting liposome suspension was further diluted to give $5.4 \cdot 10^{-5}$ M as phosphatidylcholine concentration, where the effect of light scattering on the fluorescence anisotropy measurements was negligible [19,20].

Fluorescence measurements. Fluorescence spectra were measured by essentially the same procedure as that described [15,21,22]. All the spectral measurements were run on a Hitachi 650-10S fluorospectrophotometer equipped with a thermoregulated cell compartment connected to a Toyo Thermo Electric TE-104S. Steady-state fluorescence anisotropy of DPH as a function of temperature was measured on a Union Giken fluorescence polarization spectrophotometer FS-501S, of which cell compartment was connected to a Komatsu-Yamato Coolnics Model CTR-120. A Sord Microcomputer M 200 Mark II system was combined with the instrument to control the measurement conditions and collect all the data. DPH was excited at 360 nm and its fluorescence was detected using a sharp cut-off filter L-39 (Hoya Glass Works, Tokyo) to shut out the light of wavelengths less than 370 nm.

The following relationship was used to obtained the steady-state fluorescence anisotropy, r_s, of the probe

$$r_s = \frac{I_{VV} - C_f \cdot I_{VH}}{I_{VV} + 2C_f \cdot I_{VH}} \quad (1)$$

where I is the fluorescence intensity and subscripts V and H indicate the vertical and horizontal orientations of the excitation (first) and analyzer (second) polarizers, respectively. $C_f = (I_{VH}/I_{HH})$ is the grating correction factor. The r_s values of DPH in DPPC and DMPC liposomes obtained experimentally in this work were in good agreement with those of previous findings by other research groups [10,11,23].

The steady-state fluorescence anisotropy (r_s) can be described by the following equation [13,24]:

$$r_s = \frac{r_0 - r_\infty}{1 + \tau/\phi} + r_\infty \quad (2)$$

where τ and ϕ are the fluorescence lifetime and rotational relaxation time, respectively. r_0 and r_∞ are the maximal and limiting fluorescence anisotropy, respectively, and related to the order parameter (S) of membranes in the following manner [13,24].

$$S^2 = \frac{r_\infty}{r_0} \quad (3)$$

The r_0-value has been estimated to be 0.395 by Kawato et al. [11] using a nanosecond time-resolved fluorescence technique. In addition, Jähnig [13] has recently proposed a way of evaluating the order parameter from steady-state fluorescence anisotropy measurements, and he shows that the ratio, τ/ϕ of DPH in DPPC liposomes is almost constant at 8, irrespective of the incubation temperature due to equivalent temperature dependency of both parameters. Hence, the order parameter S at a given temperature can be obtained with the aid of Eqns. 1 through 3.

Results and Discussion

Fluorescence depolarization is a potent technique for membrane research [8] and provides

information about static and dynamic properties of membranes [10–12]. Of various fluorescent probes utilized so far, 1,6-diphenyl-1,3,5-hexatriene (DPH) seems most convenient as a tool to monitor the rotational motion of the probe embedded in liposomal bilayers, since it is well examined especially by the nanosecond time-resolved fluorescence depolarization technique [10–12]. All the fluorescence spectra of DPH in liposonal membranes employed in this work were exactly the same as those observed in benzene. The shape of these spectra was not affected at all by the gel-liquid crystalline phase transition of liposomes. These findings show that the location of DPH molecules are in the hydrophobic domain of lipid bilayers [25,26], which is common in all the phosphatidylcholines employed in this work.

The steady-state fluorescence anisotropy, r_s, of DPH embedded in liposomal bilayers was measured as a function of temperature (Fig. 1). For both DPPC and DMPC liposomes, the abrupt changes in r_s were observed around their respective phase transition temperature (T_c). However, 13_{CY}PC liposome does not show a remarkable change in the r_s values at the T_c (33.5°C) determined by the differential scanning calorimetry (DSC) measurement (Endo, T., et al. (1982) Ref. 34). Since the gel-liquid crystalline phase transition of 11_{CY}PC and 1-13_{CY}-2-11_{CY}PC liposomes take place at 13 and 9°C, respectively, they were not reflected on the r_s temperature profile over our experimental temperature range. At lower incubation temperatures (< 20°) which are below the T_c of DPPC liposome, that r_s values of 13_{CY}PC are smaller than dose of DPPC (Fig. 1A). This means that the introduction of ω-cyclohexyl moiety for the straight acyl chain tends to 'fluidize' the liposomal bilayers even in the gel state. These are probably responsible for the packing of unusual phosphatidylcholines being sterically hampered in the gel state by the cyclohexyl ring bound to the terminal of acyl chains. A similar effect on fluidizing in the gel state has been observed for the fatty acids containing cyclopropane ring [27], which is commonly found in the bacterial cell membrane lipids [27,28].

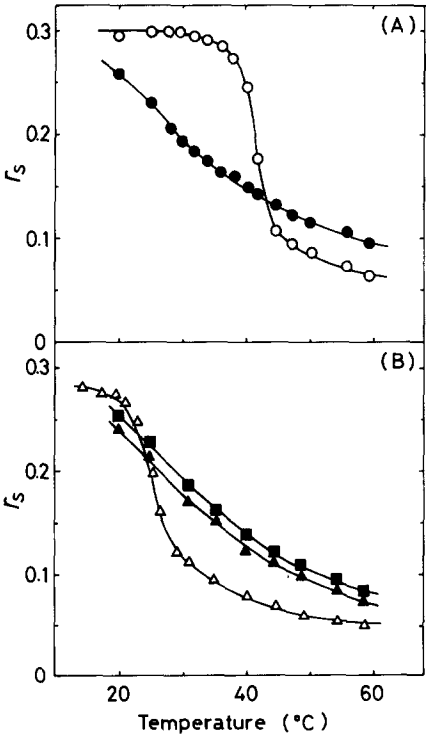


Fig. 1. Steady-state fluorescence anisotropy (r_s) of DPH in different liposomal membranes as a function of incubation temperature in 0.1 M aqueous solution. Phosphatidylcholine = $5.4 \cdot 10^{-5}$ M. (A) ○——○, DPPC; ●——●, 13_{CY}PC and (B) △——△, DMPC; ■——■, 11_{CY}PC; ▲——▲, 1-13_{CY}-2-11_{CY}PC.

Thermodynamic investigation gives much information about the membrane properties in the gel state, but not about those in the liquid-crystalline state. One the other hand, the steady-state fluorescence anisotropy provides useful information even in the liquid-crystalline state of membranes. Basically, the limiting fluorescence anisotorpy, r_∞, must be determined by the time-resolved fluorescence anisotropy measurements [10–12]. However, the procedure suggested by Jähnig [13] for estimation of the r_∞ value from steady-state fluorescence anisotropy measurements seems reliable within the limits of qualitative discussion. The order parameters, S, in our system was then estimated by the method of Jähnig [13]. As shown in Fig. 2, in the liquid-crystalline state, the order parameters of liposomal bilayers consisting of unusual phosphatidylcholines are always larger than those of regular phosphatidylcholines carrying the straight fatty acids. This dual character brought about by the cyclohexane ring, which 'immobilizes' lipids in the liquid-crystalline state while 'fluidizing' them

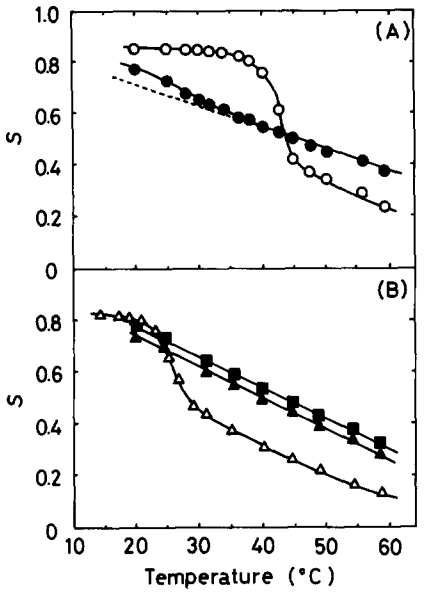


Fig. 2. Order parameter (S) of different liposomal membranes as a function of incubation temperature. (A) ○——○, DPPC; ●——●, 13_{CY}PC and (B) △——△, DMPC; ■——■, 11_{CY}PC; ▲——▲, 1-13_{CY}-2-11_{CY}PC.

in the gel state, closely resembles the phenomena observed when cholesterol was added into DPPC [29] or di-(dihydrosterculoyl)phosphatidylcholine [30] liposomal bilayers. Of the phosphatidylcholines bearing the unusual fatty acyl residue, 13_{CY}PC is the most ordered. This is closely related with previous findings that increasing growth temperature of thermophilic bacteria brings about the chain elongation of fatty acids [31].

The permeability property of liposomal membranes is also dependent on the acyl chain packing density [32]. Oshima and his coworkers [6,33] recently proposed the idea that biomembranes containing a large amount of ω-cyclohexyl fatty acids may play an important role in maintaining the internal pH neutral, since the intracellular pH is kept neutral in *B. acidocaldarius* and bacteriophage ϕNS11. Independently, three of the present authors (K.I., T.E., and S.N. unpublished data) investigated the permeability properties of 11_{CY}PC liposomes toward glucose. They adopted the light scattering technique related with the osmotic shrinking and swelling of vesicles when the concentration gradient of glucose between the exterior and interior of liposomes is generated (Endo, T., unpublished data). Table 1 clearly shows that the barrier function of DMPC liposome drastically decreases above about 40°C, while that of 11_{CY}PC is well maintained even above its phase transition temperature. Results are not inconsistent with those observed for the steady-state fluorescence anisotropy of DPH in liposomes as a function of temperature (Table 1 and Fig. 1). This means that the steady-state fluorescence anisotropy measurements are still valid to qualitatively discuss the ordering effect or packing of liposomal mem-

TABLE I

REVOVERY (%) OF SHRUNK LIPOSOMES TO RE-SWOLLEN LIPOSOMES UPON THE GLUCOSE PERMEATION BASED ON MAINTAINING THE OSMOTIC BALANCE AT VARIOUS TEMPERATURES

A mixture of phosphatidylcholine (3 μmol) and dicetylphosphate (0.3 μmol) was swollen with 10 ml of 20 mM aqueous glucose solution. Into 480 μl of the liposome suspension was added 290 μl of 1 M aqueous glucose solution and the change in the vesicle size was monitored by light scattering at 450 nm as a function of time at a given incubation temperature. Measurements were run on a Shimadzu Recording Spectrofluorophotometer RF-501. Just after the addition of glucose, liposomes first shrink upon the leakage of water from the interior to the exterior, and then the permeation of glucose into liposomes starts, which results in the re-swelling of the shrunk liposomes to maintain the osmotic balance between the exterior and interior of liposomes, which was estimated by the absorbance ratio 20 min after the addition of glucose. If liposomes showed resistance to the permeation of glucose, re-swelling could not be observed. Values are recovery ratios expressed in percent.

Liposome	Incubation temperature, °C						
	13	23	33	38	43	48	53
DMPC (T_c = 23°C)	–	100	0	–	30	41	50
11_{CY}PC (T_c = 13°C)	100	0	0	0	–	–	0

branes relating to the results from the DSC or permeability investigations. Even if the large difference in species between glucose and proton is taken into account, our present work will help towards the understanding of the necessity of the ω-cyclohexyl moiety in acido-thermophilic bacterial membrane lipids in maintaining the pH gradient across the membranes. As the next approach in the research project, we have prepared several kinds of glycolipid. The investigation on the contribution of oligosaccharide moiety as the headgroup in the acido-thermophilic bacterial membrane lipids to thermophilicity and protection from acidic environment is in progress in our laboratories.

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