A Thermotropic Behavior of Egg PC Liposome Containing the Very Long Chain Fatty Acyl Component, α,ω-13,16-Dimethyloctacosanedioate Dimethyl Ester (DME C30) Isolated from the Thermophilic Anaerobic Bacteria, *Thermoanaerobacter ethanolicus*

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Received May 24, 2001

Thermoanaerobacter ethanolicus is a strictly anaerobic and thermophilic bacterium whose optimum temperature ranges over 65-68 °C. *T. ethanolicus* was known to contain a bipolar very long chain fatty acyl component such as α,ω-13,16-dimethyloctacosanedioate as one of the major membrane components. However, exact physiological role of this unusual component in the membrane remains unknown. Such a very long chain fatty acyl component, α,ω-13,16-dimethyloctacosanedioate dimethyl ester (DME C30), was isolated, and purified from the membrane of *T. ethanolicus*. As a function of added concentrations of the α,ω-13,16-dimethyloctacosanedioate dimethyl ester (DME C30) or cholesterol into the standard liposomes, the acyl chain ordering effect was investigated by the steady-state anisotropy with 1,6-diphenyl-1,3,5-hexatriene (DPH) as a fluorescent probe. Acyl chain order parameter (S) of vesicles containing DME C30 is higher comparing with phosphatidylcholine (PC) only vesicles. This result was discussed thermodynamically with the aid of the simulated annealing molecular dynamics simulations. Through the investigation of all the possible conformational changes of DME C30 or cholesterol, we showed that DME C30 is very flexible and its conformation is variable depending on the temperature comparing with cholesterol, which is rigid and restricted at overall temperature. We propose that the conformational change of DME C30, not the configurational change, may be involved in the regulation of the membrane fluidity against the changes of external temperature.

Keywords : Very long chain fatty acid, Liposome, Membrane fluidity, Molecular dynamics.

Introduction

Thermoanaerobacter ethanolicus is a strictly anaerobic and thermophilic bacterium whose optimum temperature is 65-68 °C.[1,2] High temperature induces high degrees of motion of lipids and destabilizes the membrane. Thermophilic bacterium must maintain the optimal membrane fluidity at a high growth temperature to survive.[3] In case of *T. ethanolicus*, it has a family of unusually very long chain α,ω-13,16-dimethyloctacosanedioate as a major fatty acid component in their membrane lipids. Similar α,ω-dicarboxylic acids were also discovered in strict anaerobic, acidophilic bacterium, *Sarcina ventriculi* where a family of dicarboxylic acids were induced after the various environmental stresses which fluidize the membrane.[4,5,6] α,ω-Dicarboxylic, very long chain fatty acyl components were mostly found in extremophilic bacteria which survived harsh environmental stresses against membrane components. Their unusually long chain structures give more conformational freedom as well as stronger *van der Waal* interaction. Although their exact conformations or physiological functions inside the membrane still remain to be unknown,[2] it is believed to be responsible for the maintenance of membrane integrity against environmental heat stress.

However, no direct reports have been made on the measurements of membrane fluidity induced by the presence of very long chain α,ω-dicarboxylic components. Many studies have been performed to understand the interactions between cholesterol and either dipalmitoylphosphatidylcholine (DPPC) or egg PC. It has been demonstrated by a variety of physical techniques that cholesterol has a condensing (ordering) effect on the packing of phospholipids in the liquid-crystalline (fluid) state, *i.e.*, above the phase transition temperature.[7] In order to understand the physiological role of the α,ω-13,16-dimethyloctacosanedioate moiety in the thermophilic bacterial membrane, a synthetic model membrane was constructed from egg PC and α,ω-13,16-dimethyloctacosanedioate dimethyl ester (DME C30), after the complete isolation and purification of DME C30 from the total fatty acid methyl esters of the membrane of *T. ethanolicus*. The changes of molecular order parameter of the membrane caused by the presence of DME C30 or cholesterol were investigated using a fluorescence lipid probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) embedded in the membrane.[8,9] The dependence of the molecular order on the conformation of its unusual component was also investigated performing ten times simulated annealing molecular dynamics with the temperature change between 300 K and 1000 K at an interval of 50 K.[10]

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Experimental Section

Materials. Ninety nine % egg L-α-phosphatidylcholine (PC) was purchased from Sigma Chemical Co. and used without further purification. The α,ω-13,16-dimethyloctacosanedioate dimethyl ester (DME C30) was isolated by the procedure below. Cholesterol was purchased from Yakuri Pure Chemicals Co. and used without further purification. The fluorescence probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Sigma Chemical Co.

Isolation of α,ω-13,16-dimethyloctacosanedioate dimethyl ester (DME C30). *T. ethanolicus* was obtained from Korea Collection for Culture Type as KCTC 3184 (ATCC 31938). The cells were cultured on a complex medium that contained 0.2% (w/v) yeast extract, salts, and vitamin with 0.8% (w/v) glucose under a strictly anaerobic condition at 65 °C.[1,2] After cells (40-45 g) were harvested by centrifugation at 10,000 × g for 10 min, those were added to 400 mL 5% methanolic HCl solution and soxhlet extraction was performed at 75 °C for 24 h.[2] Chloroform (20 mL) was added every 8 h, followed by mild sonication for 5 min. The extracts were concentrated on the rotary evaporator and the mixture of fatty acid methyl esters was isolated through partitioning the residue between chloroform and water. The aqueous layer was washed several times with chloroform or hexane. The combined solutions were filtered through the glass wool. Each fatty acid methyl ester was then identified by gas chromatographic (GC) analysis on a J&W Scientific DB1 capillary column using N_2 as the carrier gas. The temperature was initiated at 150 °C, 0.00 min hold time, increased 3 °C/min rate to 300 °C. The temperature was held for 40 min. The mixtures of fatty acid methyl esters were applied to a silica flash chromatography column[11] and eluted with chloroform-hexane 1.5 : 1.0 (v/v) at a flow rate of 1 mL/sec. Fractions of partial purified fatty acid methyl esters were applied to preparative thin layer chromatography (TLC) and eluted with chloroform-hexane 1.0 : 1.0 (v/v) to obtain the pure α,ω-13,16-dimethyloctaco- sanedioate dimethyl ester (DME C30). Fractions were assayed by gas chromatography (GC) or gas chromatography/mass spectrometry (GC/MS) analysis on a Jeol JMS-AX505WA. One band containing the pure DME C30 was subjected to further analysis.

Preparation of vesicles. Egg PC was dried by N_2 gas and suspended in Tris-HCl buffer (pH 7.4) at concentrations of 5-10 mg/mL and labeled with DPH (from a 2 mM stock solution in tetrahydrofuran) to give 250 : 1 as a final concentration ratio for lipid to probe.[9,12] DME C30 or cholesterol were present at 10 mol % in the lipid vesicle. PC only vesicles were also prepared. Small unilamellar vesicles (SUV) were prepared by the sonication using a probe-type sonicator at 0 °C.

Steady-state fluorescence anisotropy measurements. All the data were obtained on a SPEX/Fluorolog-T2 spectrofluorometer. The steady-state anisotropy (r_s) is defined as a ratio $(I_{II} - I_{\perp})/(I_{II} + 2I_{\perp})$ where I_{II} and $I_{\perp}$ are the fluorescence intensities measured parallel and perpendicular to the vertically polarized exciting beam. The excitation wavelength was 350 nm and the fluorescence light was detected by using 435 nm.[8] Fluorescence anisotropy with DPH were determined in SUV suspensions stabilized at various temperatures such as 0, 23, 46, 65, and 83 °C.

Data analysis. By application of Perrin's formalism for rotational depolarization of a fluorophore, the fluorescence anisotropy r_s of DPH in membranes can be correlated with the apparent 'microviscosity' $\overline{\eta}$.[13] More precisely, the fluorescence anisotropy parameter $[(r_0/r_s)-1]^{-1}$, where r_0 is the limit anisotropy value in a rigid medium ($r_0 = 0.362$), is proportional to and for comparative purposes can be used as a relative scale.[13] This microviscosity (or inversely the fluidity) is an average term that describes the bulk properties of the lipid bilayer. According to Pottel *et al*.,[14] an acyl-chain order parameter (S) can be estimated from DPH fluorescence anisotropy data using the following equation:

$$s = \frac{[1 - 2(r_s/r_0) + 5(r_s/r_0)^2]^{1/2} - 1 + (r_s/r_0)}{2(2_s/r_0)}$$

Conformational search of DME C30 and cholesterol by simulated annealing molecular dynamics minimization simulation. Conformational search of DME C30 and cholesterol over a wide range of temperature was performed by simulated annealing molecular dynamics full minimization strategy. In the simulated annealing molecular dynamics (MD) simulation, the temperature was changed ten times between 300 K and 1000 K at an interval of 50 K. At each temperature, MD simulation was performed for 10.5 ps : 2.5 ps of equilibration phase and 8 ps of production phase. One structure was saved from the end of each production phase. Total MD simulation time was 2950.5 ps. Molecular dynamics calculation was performed with DISCOVER program (version 2000, Molecular Simulations Inc.) using consistent valence force field.[15] No cutoff was imposed on the calculation of non-bonded interactions. NVT MD calculation was performed using the leap-frog algorithm with a 1 fs time step. Temperature was controlled by the velocity scaling in equilibration phase and was determined by the Berendsen algorithm[16] in production phase with coupling constant of 0.2 ps. Dielectric constant was set to 1. After the MD simulation, all the 281 structures were fully energy-minimized: 100 iterations of steepest descent minimization and conjugate gradient minimization until the maximum derivative reached below 0.001 (typically 5,000-10,000 iteration).

Results and Discussion

Isolation of DME C30. GC and GC/MS analyses were performed after the conversion of fatty acids extracted from cells to methyl esters by acid-catalyzed methanolysis. The complete isolation of α,ω-dicarboxylic, very long chain fatty acyl components was performed. Figure 1(A) shows the TLC analysis on the isolated fractions of flash column chromatography with silica gel and Figure 1(B) shows the purified DME 30 in the GC analysis. Its exact structure was confirmed by GC/MS analysis as the α,ω-13,16-dimethyloctacosanedioate dimethyl ester (DME C30) as published before.[2]

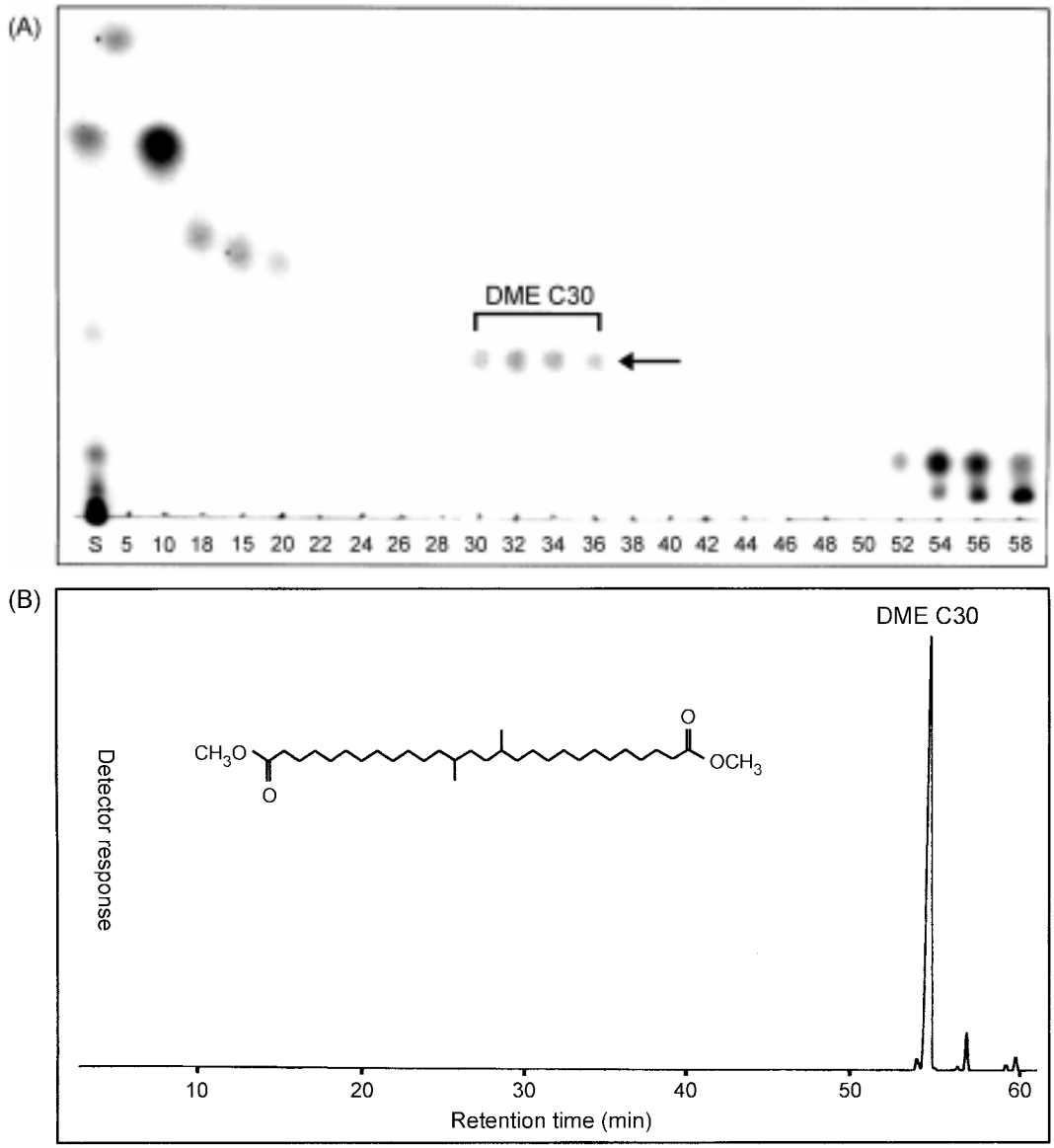

Figure 1. (A) Isolated TLC fractions (arrow) of the α,ω-13,16-dimethyloctacosanedioate dimethyl ester (DME C30) by a flash column chromatographic method. (B) Gas chromatographic analysis of the isolated and purified DME C30. Its exact chemical structure was confirmed by a GC/MS analysis.

Steady-state fluorescence anisotropy measurements. Effects of the DME C30 on the steady-state fluorescence anisotropy of DPH embedded into egg PC vesicles were compared with the egg PC only or PC/cholesterol model system at various temperatures. Table 1 showed the effect of temperature on the acyl-chain order parameter (S). The S value of liposomal vesicles containing DME C30 was higher than PC only vesicles for overall temperatures. It may act as a stabilizer or condenser for the liposomal vesicles. However, cholesterol shows the highest S value, which was known to have a condensing (ordering) effect on the packing of phospholipids in the liquid-crystalline state.[7] As the possible conformations of the DME C30 and cholesterol were important at the temperatures, computational conformational analysis was performed as followings.

Table 1. Order parameter values calculated from DPH anisotropy data for each vesicle

Lipid Composition	23 °C		46 °C	
	$((\gamma_0/\gamma_s)\text{-}1)^{-1}$	S^a	$((\gamma_0/\gamma_s)\text{-}1)^{-1}$	S^a
PC + cholesterol (10 mol%)	0.487	0.406	0.412	0.359
PC + DME30 (10 mol%)	0.401	0.352	0.323	0.295
PC only	0.362	0.324	0.287	0.267

[a]Acyl chain order parameter

Conformational analysis of DME C30 and cholesterol. In general the fluidity of a bacterial membrane is regulated mainly by the compositional change of the fatty acyl chains of the lipid.[17,18] The degree of saturation per unsaturation of fatty acyl chain or the change of a carbon chain length is an important factor for the regulation of membrane fluidity.[19,20] However, in the case of the PC vesicles containing the DME C30, the acyl

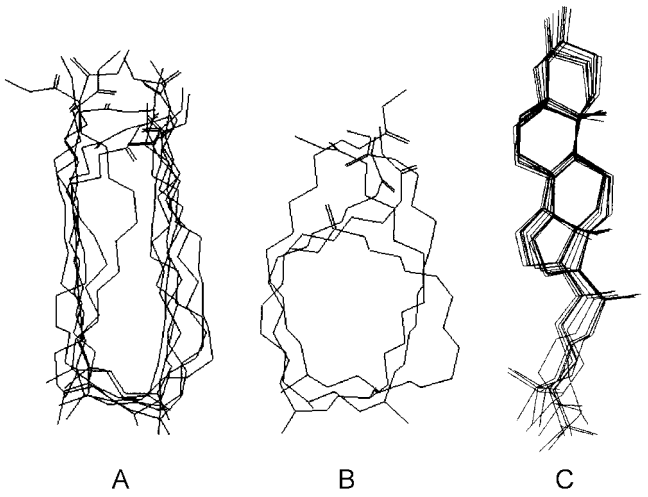

Figure 2. The conformations within 2 kcal/mol of the lowest energy minima were categorized into two classes. The conformations in each class were superimposed and represented as the lines styles; (A) seven anisotropic conformations (Radius of gyration; 5.860 ± 0.282 Å). (B) three isotropic conformations (Radius of gyration; 5.494 ± 0.162 Å). (C) ten conformations of cholesterol (Radius of gyration; 5.296 ± 0.147 Å).

chain order parameter was changed depending on external temperature. As the molecular order in membrane also depends on the conformational change of its component as well as the compositional change, we tried computationally to investigate all the possible conformations of an unusual DME C30 fatty acyl component as well as cholesterol. A simulated annealing molecular dynamics minimization calculation was ten times performed with the temperature changed between 300 K and 1000 K at an interval of 50 K. After heavy searching process of possible conformational states of the DME C30 and cholesterol, we obtained the 10 conformations within 2 kcal/mol of the lowest energy minimum categorized, respectively. Cholesterol did not show any noticeable conformational changes. Ten conformations of cholesterol were shown in Figure 2(C). The average value of radius of gyration was estimated to 5.296 ± 0.147 Å (Table 2). However, in case of DME C30, we made a further classification to two hypothetical conformational classes as shown in Table 3 as its shape. These were anisotropic (Figure 2(A)) or isotropic forms (Figure 2(B)) depending on their characteristic conformations. The average value of radius of gyration was estimated to 5.860 ± 0.282 Å for the anisotropic forms and 5.494 ± 0.162 Å for isotropic ones. Then, the anisotropic forms could increase the neighboring acyl chain order more efficiently than the isotropic ones because of its enhanced *van der Waals* interaction due to the relatively large surface area. Their conformations showed an interesting analogy of the membrane lipid structure. The anisotropic forms might be analogous to the lipids with saturated fatty acyl chains, whereas the isotropic ones with unsaturated fatty acyl chains. If we apply the Boltzmann distribution to determine the probability of finding a molecule with energy ΔE_i, the probability P_i is,

$$P_i = \frac{e^{-\Delta E_i/RT}}{\sum e^{-\Delta E_i/RT}}$$

where R is the gas constant (8.314 J/mol · K) and T is the absolute temperature (K).

We define $P_{isotropic}$ and $P_{anisotropic}$ as below;

$P_{anisotropic} = \Sigma P_{anisotropic}$, and $P_{isotropic} = \Sigma P_{isotropic}$

Boltzmann distribution was applied to the 10 conformations

Table 3. Energy of 10 conformations of DME C30 within 2 kcal/mol of the lowest energy minimum among 281 structures (frames) obtained from the MD simulation

Rank	Frame	ΔE (kcal/mol)	Conformation	R_g[b]
1	198	0[a]	anisotropic	5.272
2	195	0.10	anisotropic	5.751
3	83	0.31	anisotropic	5.870
4	143	0.72	anisotropic	6.022
5	76	0.96	isotropic	5.565
6	253	1.41	isotropic	5.308
7	84	1.46	anisotropic	6.032
8	30	1.92	isotropic	5.608
9	85	1.93	anisotropic	6.048
10	59	2.00	anisotropic	6.024

[a]The lowest energy value is 28.81 kcal/mol. [b]Radius of gyration

Table 2. Energy of 10 different conformations of cholesterol within 2 kcal/mol of the lowest energy minimum among 281 frames obtained from the MD simulation

Rank	frame	ΔE (kcal/mol)	R_g[b]
1	12, 37, 49, 51, 90, 108, 134, 146, 148, 152, 181, 237, 238, 243	0[a]	5.180
2	50, 121, 145, 147, 232	0.03	5.215
3	6, 45, 73, 81, 86, 164, 165, 167, 205, 222, 226, 228, 259, 277, 280, 281	0.53	5.506
4	84, 85, 88, 125, 160, 170, 174, 223, 224, 225, 229, 230	0.54	5.521
5	72, 93, 135, 138, 141, 142, 213, 234	0.58	5.123
6	89, 102, 106, 109, 110, 112, 113, 114, 115, 143, 144, 182, 183	0.72	5.096
7	22, 23, 43, 92, 136, 137, 153	0.85	5.281
8	5, 8, 47, 48, 57, 58, 60, 82, 83, 87, 168, 178, 202	1.08	5.322
9	4, 122, 171, 215, 276	1.21	5.374
10	34, 54, 55, 56, 69, 169, 177	1.23	5.347

[a] The lowest energy value is 92.89 kcal/mol. [b]Radius of gyration

of the lowest energy minima obtained in the simulation. $(P_{anisotropic}/P_{isotropic})_{lowT} > (P_{anisotropic}/P_{isotropic})_{highT}$ was maintained such as 8.877 at 23 °C changed to 7.912 at 46 °C. As the applied temperature increases, the relative proportion of anisotropic forms to isotropic ones decreases. It is likely that conformational change of DME C30 could regulate the fluidity of the membrane similar to the change of the ratio of unsaturation per saturation in the fatty acyl chains in the membrane lipids. The conformational changes of this unusual fatty acyl component are analogous to the configurational changes observed in the homeoviscous adaptation of the fatty acyl chains in the lipids. As a typical configurational adaptation of the lipids, the ratio of saturated fatty acids to unsaturated ones within the lipids decreased as the external temperature increased.[21]

Conclusion

Membranes are critical components of the cell surface of living organisms that form a barrier between the cell cytoplasm and its surroundings through which many metabolites must pass. Membranes must possess very sophisticated molecular mechanisms for maintaining sufficient fluidity for molecular events to take place and keeping rigidity as well for the cellular integrity.[20] Most microorganisms use homeoviscous adaptability to change the chemical properties of the membrane so as to restore the original fluidity after an environmental perturbation that changes membrane viscosity. In general, microorganisms genetically regulate the degree of unsaturation, chain length, branching, or cyclization of fatty acid to give the optimal molecular order inside the membrane.[21,22] However, molecular order of the membrane also depends on the conformational change of its components where they have unusual structural characteristics such as the DME C30.[10] DME C30 exist s only in the membrane of *T. ethanolicus* and its physiological function is still unknown. Throughout the experiments and computational speculations, we tried to elucidate the possible role of the DME C30 as a fluidity regulator responding to the change of external temperature. With the aid of computational simulation, we propose a hypothesis that conformational change of DME C30, not the configurational change, makes it possible for a thermophilic membrane to have its optimal viscosity and this is probably one of the physiological roles of unusually long chain fatty acids existing in *T. ethanolicus.* In this regard, further investigation will be needed on the whole lipids isolated from *T. ethanolicus*.

Acknowledgment. This research was supported by a grant No. KOSEF 981-0513-064-2 from the Korea Science and Engineering Foundation. SDG.

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