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TEMPERATURE SCANNING X-RAY DIFFRACTION AT PHASE TRANSITIONS OF BIOLOGICALLY RELATED LIPID ASSEMBLIES
Special attention to cholesterol-rich state

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Based upon the results of ac calorimetry and temperature scanning X-ray diffraction in the phospholpid/cholesterol system, the phase diagram was constructed by taking into account the ripple structure. From the analysis of the cholesterol concentration dependence of the modified ripple structure the cholesterol-rich state which lies in the higher cholesterol concentration than 20 mol% in the phase diagram is proposed. It is proposed that this is a fundamental complex that appears generally in lipid/cholesterol systems.

Keywords: *ac calorimetry, cholesterol, liquid-ordered phase, phase diagram, phospholipid, raft, ripple phase*

Introduction

In biomembrane, microdomains such as a raft, which is enriched in sphingolipids as well as cholesterol (CHOL), have been proposed as organizing platforms for proteins with certain kinds of lipid anchors, including signaling proteins. To consider the formation of a raft in biomembrane, phase diagram of a phospholipids/cholesterol system has played an important role, since based upon the phase diagram the concept of a liquid-ordered phase has been established in this system and it is possible to consider further the interaction between lipid and CHOL in the raft [1]. So far, a lot of studies have been performed in the systems of CHOL and synthetic phospholipids where it should be stressed that a couple of the acyl chains is saturated as in sphingolipids.

The interaction between phospholipid and CHOL is still a subject attracting the interest of many researchers. Based upon X-ray diffraction [2] and calorimetry [3] in the CHOL-rich state the arrangement of phospholipid and CHOL molecules has been discussed. In this system, in the upper view composed of the lateral arrangement of cross sections of a hydrocarbon chain and a CHOL molecule, a CHOL molecule is surrounded by a small number of phospholipid molecules so as not to form CHOL-CHOL contact [2]. In the side view composed of the side-by-side arrangement of hydrocarbon chain and long CHOL molecule the molecular interaction has been discussed from molecular dynamics for the system of CHOL and fatty acid [4]. For the fatty acids around a CHOL molecule, in the one side layer of a bilayer the upper half of the acyl chain is in contact with the rigid steroid ring, which reduces the conformational degree of freedom in the acyl chain, whereas the lower part is mainly in contact with the flexible CHOL tail, which allows kinks in the acyl chain. Such behavior of the acyl chains is consistent with the behavior that the order parameter drops significantly toward the methyl end of the acyl chain. Furthermore, from a molecular dynamics simulation of a dimyristoylphosphatidylcholine (DMPC)/CHOL system containing about 20 mol% CHOL it has been found that the packing of hydrocarbon chains in DMPC on the smooth α-face side of the CHOL ring is similar to that of the pure DMPC bilayer in the gel phase and on the other hand the packing on the rough α-side is less regular and much loose [5].

In the CHOL-rich state a CHOL molecule affects the dynamics of phospholipid molecules in membranes. It has been generally accepted that in the gel phase the incorporation of CHOL molecules into a phospholipid bilayer results in fluidization of the acyl chains and on the other hand in the liquid-crystalline phase results in decrease of motional degree of freedom in the acyl chains. This is called dual effects of CHOL on the fluidity in the phospholipid membranes [6]. The concept of the dual effects is important until now because the characteristic of the rafts is strongly related to them.

In the present paper, based upon the phase diagram of the phospholipid/CHOL system which was obtained from the analyses of the ac calorimetry and the temperature scanning X-ray diffraction, I will propose a complex composed of phospholipid and CHOL for the CHOL-rich state.

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Proposal of a cholesterol-rich state

To understand the thermodynamic behavior of the phospholipid/CHOL system, it is useful to study the phase diagram. Generally a calorimetric study is powerful to construct the phase diagram. From high-sensitive differential scanning calorimetry (DSC) [7, 8] and ac calorimetry [9], it has been found that the thermogram near the main transition is composed of two anomalies. The ac calorimetry is superior to the other calorimetries in observing the detailed behavior of the heat capacity anomaly. In fact, the ac heat capacity [9] in a DMPC/CHOL system takes place a sharp peak at the main transition (that is, the gel to liquid-crystalline phase transition) of DMPC and additionally appears a broad but clear peak at the higher temperature. As increasing the cholesterol concentration, the sharp peak becomes small and on the other hand the broad peak increases in the beginning, turns to decrease, and finally diminishes at the 20 mol% CHOL in the DMPC/CHOL system. The diminishment happens at the same time in both the sharp and the broad peaks in the DMPC/CHOL system. In a dipalmitoylphosphatidylcholine (DPPC)/CHOL system the ac heat capacity changes almost in the same manner as in the DMPC/CHOL system with increasing the CHOL concentration. The sharp peak at the main transition also diminishes at the 20 mol% CHOL, but the broad peak remains up to the 50 mol% CHOL [9]. Here it is worth while to point out that by DSC essentially the same behavior has been observed in a sphingomyelin/CHOL system [10, 11]. To study the interaction between lipid and CHOL it is of interest to note the above behavior that depends on the carbon number of the acly chains in the phospholipids. I will point out later on that the typical complex between phospholipid and CHOL is formed in the DMPC/CHOL system in which the carbon number of the acyl chain of DMPC is fourteen.

From the results of ^{2}H nuclear magnetic resonance in addition to DSC, Vist and Davis [12] have proposed the phase diagram of the phospholipid/CHOL system, in which there are three phases; the liquid-crystalline phase, the gel phase and the CHOL-rich phase. For the above experimental result, Ipsen *et al*. [1, 13] have interpreted theoretically these phases in terms of a solid-ordered phase (so), a liquid-disordered phase (ld) and a liquid-ordered phase (lo), respectively. The concept of the lo phase, in which CHOL disturbs the translational order in the solid (gel) state of phospholipids, has been pointed out at the first time in their study. The lo phase appears above a certain CHOL-concentration which is 20 mol% in the DMPC/CHOL system. Therefore a phase boundary takes place at the 20 mol% CHOL in the DMPC/CHOL system (generally as shown by the vertical line at c_c in Fig. 1). For the pure phospholipid system the ld phase lies above the main transition (T_m in Fig. 1). For the phospholipid/CHOL system there is a phase boundary given by the horizontal line at T_m and above the phase boundary there is a two phase region between the ld phase and the lo phase (see a laterally bended campanulate region in Fig. 1). The ld phase connects with the lo phase in the high temperature region, that is, the reentrant behavior takes place between the ld and the lo phases. The temperature region where the broad anomaly of the heat capacity appears in the ac calorimetry [9] corresponds to the two phase region denoted by ld+lo in Fig. 1. In the DMPC/CHOL system the two phase region is almost terminated at the 20 mol% CHOL and on the other hand in the DPPC/CHOL the two phase region is extended up to the 50 mol% CHOL. The dual effects of CHOL [6] which appears at the higher CHOL concentration than the CHOL concentration at the end of the two phase region are explained by the characteristic of the liquid-ordered phase.

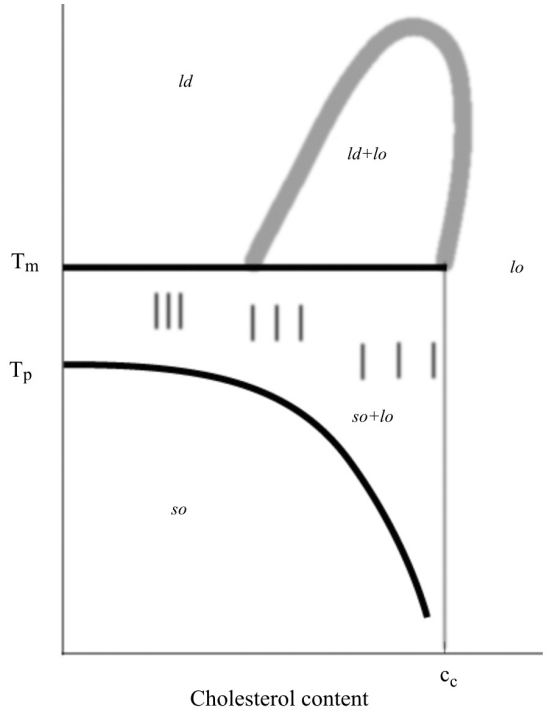

Fig. 1 Phase diagram of a phospholipid and cholesterol system constructed based upon the results of ac calorimetry and temperature scanning X-ray diffraction. Notation so is the solid-ordered phase, ld is the liquid-disordered phase and lo is the liquid-ordered phase. T_m is the main transition and T_p is the pretransition. In the modified ripple phase denoted by so+lo, the periodic arrangemnent of the ripple ridge and the flat region is schematically drawn by vertical bars. The repeat distance becomes large with the increase of the cholesterol concentration and at the critical concentration c_c reaches infinite

However, the above phase diagram of the phospholipid/CHOL system is insufficient, since the rip-

ple phase which appears in the pure phospholipid system is not taken into account. I will propose a phase diagram which includes the ripple phase as shown in Fig. 1. Temperature dependence of the ripple structure has been studies by temperature scanning small angle X-ray diffraction as a function of CHOL concentration [14]. As schematically shown in Fig. 1, below the horizontal line at T_m in the ripple phase the repeat distance increases with the CHOL concentration and at the critical concentration c_c it becomes infinite. Such a ripple structure incorporating CHOL is called a modified ripple structure hereafter. The phase diagram is essentially the same as that developed by Vist and Davis [11] except for the existence of the ripple phase. Since in the present phase diagram the ripple phase is surrounded by so, lo, ld and ld+lo in the phospholipid/CHOL systems as seen in Fig. 1, it is likely that a key factor to consider the presence of a CHOL-rich state lies behind the formation of the modified ripple structure.

As discussed above, the lo phase takes place above the critical CHOL concentration of c_c. In the ac calorimetry for the DMPC/CHOL system with increasing the CHOL concentration at the 20 mol% CHOL the sharp peak at T_m disappears and at the same time the broad peak above T_m disappears. On the other hand, for the DPPC/CHOL system, the sharp peak at T_m disappears also at the 20 mol% CHOL, while the broad peak above T_m remains up to the 50 mol% CHOL. These facts indicate that CHOL incorporated into the phospholipid membrane results in the formation of a stable complex with the 20 mol% CHOL, i.e., the appearance of the CHOL-rich state. This is the fact in the DMPC/CHOL system, while in the DPPC/CHOL system an additional contribution remains. This might be due to the phenomena that in the DMPC/CHOL system the molecular lengths in DMPC and CHOL match with each other, while the molecular length of DPPC is longer than that of CHOL. Therefore in the DPPC/CHOL system after the formation of the complex with the 20 mol% CHOL the conformational degree of freedom in a hydrocarbon chain remains and at the 50 mol% CHOL the entire degree of freedom of a hydrocarbon chain in DPPC freezes.

Discussion

For the ripple structure of the pure phospholipids a lot of studies have been performed using X-ray diffraction, freeze-fracture electron microscopy, atomic force microscopy, etc. Besides them, the electron spin resonance (ESR) and the magnetic resonance studies in the ripple phase shed light on the molecular state in the membrane [15]. The ESR spectrum due to the stearic acid spin probe embedded in the phospholipid bilayer has been explained in terms of the superposition of ordered and disordered spectra, that is, the ripple structure is composed of the two distinct states, that is, the gel state and liquid-crystalline state. From the analysis of the ESR spectrum it has been found that the ratio of the gel state and the liquid-crystalline state is estimated to be 4:1. Based upon these results, a model for the ripple structure has been proposed [15]. In the model, the ripple structure results from the banded structure in which the two bands take place periodically, that is, one major band is formed by the gel state and the other minor band is formed by the liquid-crystalline state. In the phospholipid bilayer, the modulation of the periodic structure in the one layer is different from that of the other layer by phase angle π and as a result the sinusoidal modulation of the bilayer seems to be caused. For the ripple structure, the intensive studies have been preformed by the X-ray diffraction in the phospholipids. In a widely accepted structure, the acyl chains are tilted with respect to the plane of the bilayer and the surface of the bilayer is modulated sinusoidally [16]. However, the recent detailed analysis of the ripple structure indicates that the surface of the bilayer has an asymmetric triangular ripple shape and furthermore the electron density in the acyl chains is not uniform but modulated [17]. Moreover, the recent atomic force microscopy observation shows a simple sinusoidally modulated ripple structure [18]. Then, for the ripple structure the problems which should be solved remain still. In the following, I will consider further within the framework of the evidence obtained from ESR.

From the freeze-fracture electron microscopy study on the modified ripple structure in the DMPC and CHOL system by Copeland and McConnell [19] it has been found that by incorporation of CHOL molecules into the bilayer a ripple ridge which consists of a single period of the ripple-repeat structure remains and on the other hand, between the neighboring ripple ridges a flat region that becomes wide with increasing the CHOL concentration appears. As a result, the repeat distance increases. This is consistent with the result of the small angle X-ray diffraction as mentioned above [14]. From the analysis of the relation between the periodicity and the CHOL concentration a model has been proposed, that is, the part in the gel phase is formed by a ripple ridge of pure DMPC and a flat region with the 20 mol% CHOL and the periodic arrangement of the ripple ridge, which has a character of the so phase, and the flat region, which has a character of the lo phase, results in the modified ripple structure with long period in which the so and the lo phases coexist. This fact indicates that the incorporation of even a small amount of CHOL molecules into

a DMPC bilayer forms a cluster or domain of the complex with the 20 mol% CHOL in the DMPC and CHOL system. At the 20 mol% CHOL, in the DMPC/CHOL system the overall phospholipids bilayer is cover by the flat region. Then, the phase boundary between lo and so+lo is drawn by a vertical line at the critical concentration of the 20 mol% CHOL in Fig. 1.

Conclusions

I propose that the stable CHOL-rich state (lo) of the phospholipid and CHOL system is composed of the complex with 20 mol% CHOL. I further propose that such a structure appears not only in the phospholipid/CHOL system but also generally in the lipid/CHOL system including in the sphingolipid/CHOL system, as far as the hydrocarbon chains in the lipids are saturated.

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