Free energy profiles of lipid translocation across pure POPC and POPC/CHOL bilayer: all-atom molecular dynamics study

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Abstract. All-atom molecular dynamics (MD) simulations of pure 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer and POPC bilayer containing cholesterol (POPC/CHOL) with CHARMM36 force field were carried out to investigate the effects of CHOL on structure and lipid translocation across the membranes. We calculated the potential mean force (PMF) profiles for translocation of POPC and CHOL along the pure POPC and the POPC/CHOL bilayer normal by umbrella sampling method. The obtained PMF profile of the POPC translocation was in good agreement with that of the previous MD study, showing that the estimated PMF profiles with the CHARMM36 force field should be reasonable. We found that the PMF peak appears slightly beyond the bilayer center. The CHOL effects on the PMF profile of POPC translocation were clearly observed; the free energy barrier for the flip-flop of lipid increased and, the energy for the desorption of lipid decreased. These changes in the PMF profile should be responsible for the tight packing of the POPC/CHOL bilayer. The PMF profiles for the CHOL translocation showed that the free energy barriers at the bilayer center were sufficiently smaller than those of POPC translocation, indicating that the CHOL can easily flip over compared to the POPC in the membrane. In the case of PMF of CHOL translocation in the POPC/CHOL system, while the PMF energy in the hydrophobic core region was higher than that of the pure POPC bilayer, the free energy difference at bilayer center was small.
1. Introduction

Biomembrane is a kind of membrane to separate boundary in organelle or cell and has important functions such as selective permeation of ions/molecules and activation of the membrane proteins. The biomembrane mainly consists of amphiphilic molecules such as lipids and forms bilayer structure, and the structure, dynamics and molecular permeability of the membrane depend on the kinds of lipids and lipid composition in the membrane. The cholesterol (CHOL) is an example molecule which changes the structure and dynamics of the membrane. It has been known that the orientation of lipid chain increases by adding CHOL to the lipid bilayer and changes the membrane state to the liquid-ordered phase above 20-30 mol% of cholesterol [1, 2]. The dynamics of lipid transports in the membrane such as the flip-flop changes in the lipid bilayer containing CHOL [3]. Also, cholesterol blocks spontaneous insertion of membrane proteins into liposomes [4]. Since these dynamical properties of lipids is related to important properties such a membrane fusion, the effect of CHOL on the dynamical structure and transport properties of lipids or CHOL along the membrane normal should be investigated in detail. However, due to the difficulty of experimental observation, the details of these properties are not well known yet.

In the computational studies, potential mean force (PMF) profiles of the lipid translocation across the membrane has been investigated by molecular dynamics (MD) simulations [5-8]. The calculated PMF profiles showed the large energy barriers for the flip-flop and desorption of lipids from the membrane, and these results were qualitatively consistent with the experiment observation of lipid transfer from solution to micelle [5]. In their MD studies, they have adopted the united atom model, which is a sort of coarse-grained particle model, with GROMACS force field [5-8]. On the other hand, the CMARMM force field has been standardly used for the all-atom MD simulations for lipid bilayer systems [9-11]. The MD simulations for the several kind of lipid bilayers including CHOL with the CHARMM36 force filed have been verified so far [9-11], and the estimated membrane structures such as area per lipid and order parameters have been consistent to those of experimental observations. However, in our knowledge, the free energy changes related to the lipid translocation across the membrane have not been investigated yet.

In this study, we thus carried out MD simulations of the pure 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer and the POPC bilayer containing CHOL (POPC/CHOL) with the CHARMM36 force field and calculated the PMF profiles for the POPC translocation along the pure POPC and POPC/CHOL bilayer normal. The effects of CHOL on structure and lipid translocation across the membranes were investigated in detail. We carefully verified the POPC flip-flop level position around the bilayer center by long-term position constrained MD simulations. The PMF profiles of CHOL across the pure POPC bilayer and the POPC/CHOL bilayer were also estimated to investigate the differences with those of POPC translocation and the effects of CHOL on the PMF profile.

2. Material and method

2.1. Molecules and membrane systems

The molecular structures of the POPC and CHOL are shown in Figure 1. POPC has a 16:0 palmitic acid (PA) and an 18:1 oleic acid (OA) linking to the glycerol backbone and has a phosphorylcholine (PC) head group. CHOL has a rigid sterol ring and a short hydrocarbon chain. A hydroxyl group is linked to the sterol ring of CHOL, and this hydroxyl group is oriented to the water bulk region of the membrane. Initial bilayer structures of the pure POPC and the POPC/CHOL mixtures for MD simulations consist of 128 lipids and 8,192 water molecules. In the POPC/CHOL bilayer, thirteen POPC were replaced to CHOL in each leaflet of the bilayer, corresponding to about 20 mol % of CHOL addition. We also prepared the POPC bilayer containing only one CHOL to investigate the PMF profile of CHOL across the POPC bilayer. In this system, one CHOL was replaced to POPC in the upper leaflet of the POPC bilayer. The initial coordinates of each system were prepared by using CAHRMM-GUI [11].
2.2. Molecular dynamics simulations

MD simulations of the pure POPC bilayer and POPC/CHOL bilayers were carried out for 200 ns under the constant temperature ($T = 303.15$ K) and pressure ($P = 1$ atm) condition. Nose-Hoover thermostat [12, 13] and Parinello-Rahman type barostat [14] were used to control the system temperature and the pressure. The periodic lengths of thermostat and barostat were both 1 ps. CHARMM36 force field [9, 10] and TIP3P water model [15] were adopted for lipids and water molecule, respectively. Cutoff for the van der Waals (vdW) interaction was applied using a switching scheme, within a range of radius from 10 to 12 Å. Particle mesh Ewald (PME) method [16] was adopted for the calculation of the coulomb electrostatic interactions. The time step for numerical integration of equations of motion was 2 fs, and the coordinates of the systems were stored every 1 ps. Figure 2 shows the area per molecule $A_m$ as a function of MD time for each system. $A_m$ at each MD step was calculated by dividing the $xy$ area of MD box by the lipid number in the leaflet of bilayer. The results showed that all molecular areas were sufficiently equilibrated after 100 ns. Thus, the last 100 ns of the MD runs were used for the structure analysis of the membranes. All MD simulations were done by GROMACS 5.1.2 [17].

2.3. PMF calculations

PMF profiles for the translocation of POPC and CHOL along the membrane normal were evaluated by the umbrella sampling method [18]. In this study, the reaction coordinate for the PMF calculations was defined as the difference between the $z$ elements of picked atom and the center of mass of the bilayer. The region for the umbrella sampling calculations ranges from the water bulk region ($z = 45$ Å) to the region beyond the bilayer center ($z = -5$ Å). The spring constant of harmonic potential for the position restrain was $3000$ kJ/mol/nm$^2$ and the window width was 1 Å.

First, one POPC was selected in the upper leaflet of the bilayer and the carried out the MD simulations to sample the POPC coordinates along the reaction coordinate. The harmonic potential was applied to the phosphor (P) atom of the POPC, and the picked P atom was transferred with the
velocity of 1 Å/ns from the equilibrium position of P atoms in the upper leaflet to the bilayer center and to the water bulk region direction. In this study, to carefully verify the z level position where the transferred POPC actually flips over in the membrane, 100ns term MD simulations with the position restrain were run at each z level position. This long-term MD simulations were run first from $z = 0$ Å, and the last snapshot coordinate of the MD simulation was then used as the initial coordinate for the MD simulation at the next z level position (ex. $z = -1$ Å). We finally prepared 50 initial coordinates that the P atom of the picked POPC is on each z level. Each window was equilibrated first for 10 ns, and the production runs for each window were carried out for 100 ns. The PMF profiles as a function of the reaction coordinate z were calculated by the weighted histogram analysis method (WHAM) [18]. The energy peak of the obtained PMF around the bilayer center corresponds to the free energy barrier for flip-flop, and the PMF energy in the water bulk region can be seen as the desorption energy of the lipid from the membrane.

The PMF profiles of CHOL translocation across the pure POPC and the POPC/CHOL bilayer were also evaluated to investigate the translocation properties of CHOL. We applied the same harmonic potential to the oxygen atom of the hydroxyl group of the picked CHOL in this case. The calculations of PMF profiles were done by the g_wham program [18] in the GROMACS 5.2.1 package.

3. Results and discussions

The average area per molecule $A_\per$, membrane thickness $d_\per$, and order parameter $-S_o$ for each system were listed in Table 1. The estimated $A_\per$ of pure POPC bilayer was 63.5 Å, which is comparable with experimental value, 68.3Å [19]. The $d_\per$ were calculated as the time averaged distances between z elements of the P atoms in each leaflet of the bilayer. The order parameters $-S_o$ shown in Table 1 were estimated by averaging order parameters of the two acyl chains ($sn$-1 and sn-2) of POPC in each system. The results showed that the order parameter $-S_o$ and the membrane thickness $d_\per$ increased by adding CHOL. These structural changes were also observed in the previous experiment and the MD studies [20], showing that our MD simulations with the CHARMM36 force field reproduced the relevant membrane structures for each system.

Table 1. The average area per molecule $A_\per$, membrane thickness $d_\per$, and order parameter $-S_o$ for pure POPC and POPC/CHOL bilayer. The parenthesis shows the standard deviations of the values. The listed $-S_o$ in the table were average values of $-S_o$ at each carbon of sn-1 and sn-2 chains.

|          | $A_\per$ [Å] | $d_\per$ [Å] | $-S_o$ |
|----------|--------------|--------------|--------|
| Pure POPC| 63.5 (1.3)   | 39.1 (0.7)   | 0.15   |
| POPC/CHOL| 51.8 (1.1)   | 43.3 (0.7)   | 0.21   |

![Figure 3. PMF profile of POPC across the pure POPC and POPC/CHOL bilayer.](image1)

![Figure 4. Cavity distribution profiles as a function of reaction coordinate z.](image2)
Figure 3 shows the PMF profiles for POPC translocation as a function of reaction coordinate $z$. The minimum of the PMF profile at $z = 19.4$ Å corresponds to the equilibrium level position of P atoms in the upper leaflet of the bilayer, and the PMF energy at this $z$ was defined as zero in this study. The PMF energy increases as the POPC transfers from the equilibrium position and reaches the plateau region in the water bulk region ($z > 40$ Å).

In this study, the PMF energy at the center of bilayer is given as $AG_{\text{m}}$. In the previous MD studies, the free energy at the bilayer center was assumed as the primary free energy barrier for flip-flop of lipids in the membrane [5-8]. However, the POPC does not always flip over at $z = 0$ Å where the P atom of picked POPC is on the bilayer center. In fact, our long-term MD simulation shows that the picked POPC did not flip over at $z = 0$ Å; it flipped over slightly beyond the bilayer center in this study. The peak of PMF was found at $z = -1.4$ Å and the corresponding free energy barrier from the equilibrium level position, $AG_{\text{m}}$, was 94 kJ/mol. The free energy difference at the bilayer center $AG_{\text{m}}$ was 83 kJ/mol, and this result was close to that of the previous study, 89 kJ/mol [8]. On the other hand, the desorption energy of POPC $\Delta G_{\text{desorp}}$ was 84 kJ/mol, being in good agreement with the previous study [8]. We thus conclude that the estimated PMF profiles with the CHARMM36 force field should be reasonable results.

Next, we discuss the effect of CHOL on the POPC translocation in the membrane. The PMF profile of POPC across the POPC/CHOL bilayer showed that the free energy barrier for flop-flop, $\Delta G_{\text{flop}}$, increased and, the energy for desorption of lipid, $\Delta G_{\text{desorp}}$, decreased. These PMF changes were also shown in the previous MD study for the DPPC/CHOL mixture bilayer [6]. The peak of PMF profile was found at $z = -2.4$ Å, and the energy barrier for flip-flop, $\Delta G_{\text{flop}}$, was 120.7 kJ/mol. The desorption energy $\Delta G_{\text{desorp}}$ was 76 kJ/mol. As shown in the structural analysis of the membranes, since the membrane structures such as the order parameter, area per molecules and membrane thickness changed by adding CHOL to the POPC bilayer, the changes of the PMF in the POPC/CHOL bilayer should also be responsible for the cholesterol condensing effect in the membrane. We thus analyzed cavity distribution along the bilayer normal to investigate the molecular packing in each system. For this calculation, the systems were divided into grid with size of 1 Å at each MD step, and those grids were checked if atom exits or not in the bins. Figure 4 shows the cavity distribution profile in the pure POPC and the POPC/CHOL bilayer along $z$ coordinate of the system. In this figure, the equilibrium position of P atoms in the upper leaflet of the bilayer is defined as $z = 0$ Å. The bilayer center and the water bulk region correspond to around $z = -20$ Å and 20 Å, receptivity. We found that the cavity distribution in the POPC/CHOL bilayer largely decreased around the hydrophobic core region of the membrane. The molecular packing of the POPC/CHOL bilayer thus increase, resulting in the increase of the PMF energy in the corresponding region.

![Figure 5. Molecular length of the picked POPC as a function of reaction coordinate $z$.](image)
Next, we discuss the free energy differences of $\Delta G_{\text{des}}$ between the pure POPC and the POPC/CHOL bilayer systems. We considered that the POPC structures transferred to the water bulk region changes, and these changes of lipid conformations should contribute to the changes of PMF profile. We thus plotted the molecular length of the picked POPC as a function of reaction coordinate $z$ in Figure 5. In this figure, the molecular length of POPC is defined as the distance between the P atom and the sn-2 chain tail, and the reaction coordinate $z$ is defined as the difference between $z$ elements of the P atom of the picked POPC and the equilibrium level position of P atoms in the upper leaflet of bilayer. The molecular length of the POPC slowly increased until the membrane surface region, and it then suddenly decreases when the picked POPC came out from the membrane surface. If once the POPC completely came out from the membrane, the interaction between the picked POPC and the bilayer becomes less, resulting in the plateau shape of the PMF profile in the water bulk region. On the other hand, we found that the molecular length of POPC in the POPC/CHOL bilayer decreased at lower $z$ level. In the case of POPC/CHOL bilayer, since the highly order lipid chains are tightly packed in the hydrophobic, the structural entropy should be smaller than that of the normal POPC bilayer. The disorder chain tail of POPC is thus thought to come out at smaller $z$ level to avoid the entropic cost [6].

![Figure 6. PMF profile of CHOL across the pure POPC and POPC/CHOL bilayer.](image)

Figure 6 shows the PMF profiles of CHOL across the pure POPC and the POPC/CHOL bilayer. The result shows that the free energy barrier at the bilayer center, $\Delta G_{\text{des}}$, for the pure POPC bilayer was 19.4 kJ/mol, which is sufficiently smaller than that of POPC, 83 kJ/mol, indicating that CHOL easily can flip over than POPC in the membrane. These results are corresponding the experiments [21] and previous MD study for the DPPC/CHOL bilayer [6]. The CHOL has a smaller head polar group and molecular size compared to the POPC, and these differences should be factors for the smaller $\Delta G_{\text{des}}$. On the other hand, the evaluated free energy barrier for desorption of CHOL from the membrane, $\Delta G_{\text{des}}$, was 70 kJ/mol, showing comparable free energy barrier to that of POPC, 84 kJ/mol. In the POPC/CHOL bilayer system, the PMF profile in the membrane inertia region was steeper than that for the pure POPC bilayer, and a small PMF peak was observed around $z = 6$ Å. Since the $z$ region where the PMF profile increases corresponds to that the cavity distribution largely decreases in the hydrophobic core of the bilayer (cf. Figure 4), this energy increase should also be responsible for the tight packing of POPC/CHOL bilayer. The free energy then gently decreased as the CHOL approached to the bilayer center. We found that the free energy difference at bilayer center $\Delta G_{\text{des}}$ was small, indicating that effect of CHOL on the transferring of CHOL across the bilayer center should also be small.

4. Conclusion

We carried out all-atom molecular dynamics (MD) simulations of the pure POPC and the POPC bilayer containing cholesterol (POPC/CHOL) with the CHARMM36 force field and investigated the
effects of CHOL on structure and lipid translocation along the membrane normal. The MD simulations showed that the relevant cholesterol condensing effects such as the increase of order parameter of lipid chains and the tight packing in the POPC/CHOL bilayer. We calculated the PMF profiles of POPC and CHOL translocation along the pure POPC and POPC/CHOL bilayer normal by umbrella sampling method. The obtained PMF profile of POPC across the pure POPC bilayer was in good agreement with that of the previous MD study [8], showing that the PMF profiles with the CHARMM36 force field should also be reasonable. We carefully verified the level position of flipping position of POPC around the bilayer center by long-term MD simulations and found that the PMF peak appears slightly beyond the bilayer center. The CHOL effects on the PMF profile of POPC transport across the membrane were clearly observed; the free energy barrier for lipid flipping increased and, the energy for desorption of lipid decreased. The changes of PMF in the POPC/CHOL bilayer should be due to the tight packing of lipid chains in the hydrophobic core of bilayer. The PMF profiles of CHOL across the pure POPC and the POPC/CHOL bilayer were also estimated to investigate the differences with those of POPC translocation and the effects of CHOL on the PMF profile. The obtained PMF profiles of CHOL in each system showed that the free energy barrier at the bilayer center were sufficiently smaller than those of POPC, indicating that CHOL can easily flip over than POPC in the membrane. In the case of PMF profile of CHOL across the POPC/CHOL system, while the PMF energy in the hydrophobic core region is higher than that of the pure POPC bilayer, the free energy difference at bilayer center was small.

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