

Exploring Free Energy Landscapes for Protein Partitioning into Membrane Domains in All-Atom and Coarse-Grained Simulations

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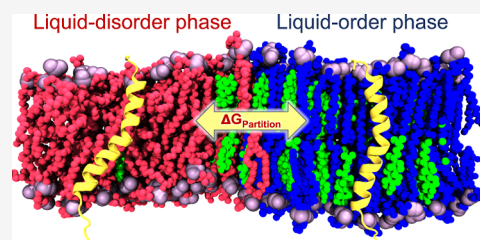


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ABSTRACT: It is known that membrane environment can impact the structure and function of integral membrane proteins. As such, elucidation of the thermodynamic driving forces governing protein partitioning between membrane domains of varying lipid composition is a fundamental topic in membrane biophysics. Molecular dynamics simulations provide valuable tools for quantitatively characterizing the free energy landscapes governing protein partitioning at the molecular level. In this study, we propose an efficient simulation methodology for the calculation of free energies for the partitioning of transmembrane proteins between liquid-disorder (L_d) and liquid-ordered (L_o) domains in all-atom (AA) phase-separated lipid bilayers. The computed potential of mean force defining the equilibrium partition coefficients is compared for AA and coarse-grained systems. Energy decomposition is used to identify differences in the underlying thermodynamics. Our findings highlight the importance of employing AA models to accurately estimate relevant free energy changes during protein translation between membrane domains.



INTRODUCTION

The compositional diversity of lipid membranes is a crucial factor that regulates the membrane's lateral organization, leading to the formation of distinct membrane domains enriched in specific lipid components and proteins.^{1–3} Among these domains, lipid rafts, which are microdomains enriched in sphingolipids and cholesterol, are believed to play critical roles in cellular processes such as protein trafficking and signaling^{4–7} by preferentially recruiting and colocalizing specific proteins. Consequently, understanding the thermodynamics that govern protein partitioning in the membrane is of fundamental importance in membrane biophysics. Numerous experimental and simulation studies have been conducted to investigate this phenomenon.^{8–11}

The coexistence of liquid-disordered (L_d) and liquid-ordered (L_o) domains in model membrane systems has been utilized to study protein partitioning into raft domains.^{12–15} In comparison with L_d domains enriched in unsaturated lipids,¹⁶ L_o domains enriched in saturated lipids and cholesterol are characterized by a relatively high degree of lipid tail order, lower area per lipid, and greater degree of lateral hexagonal packing.^{17,18} Fluorescent imaging experiments^{15,19–21} of giant unilamellar vesicles (GUVs) or giant plasma membrane vesicles (GPMVs) have provided insight into the thermodynamic driving forces that define protein partitioning between domains. It has been proposed that a protein's affinity for lipid rafts depends on several features, including the hydrophobic length of the transmembrane (TM) domain,^{13,22,23} palmitoylation,^{24–26} and surface area.¹³ Despite intensive investigation using experiments and computations, we lack a unified

framework for quantitative prediction of the thermodynamics of protein partitioning.^{19,20}

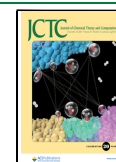
Molecular dynamics (MD) simulations offer a valuable tool for quantitatively assessing thermodynamic driving forces defining protein partitioning. Ternary lipid bilayers composed of saturated lipids, unsaturated lipids, and cholesterol undergo phase separation into L_o and L_d domains at intermediate cholesterol concentrations (10 to 40 mol %).²⁷ As such, bilayers formed from ternary lipid mixtures serve as a minimal model system for studying protein partitioning. However, one caveat of MD simulations of phase separation in lipid bilayers is that the occurrence of phase separation is dependent on the size of the simulation system. Our previous research has shown that the bilayer only exhibits phase separation above a critical system size.¹⁸ In addition, as the system size increases, the structural differences in lipids between the L_d and L_o domains become more pronounced.^{18,28} Finally, systems of sufficient size to form thermodynamically phase-separated states are challenging to perform using all-atom (AA) MD simulations due to computational costs. For these reasons, coarse-grained (CG) force fields such as MARTINI^{29,30} are often used to investigate phase-separated bilayers and protein partitioning.

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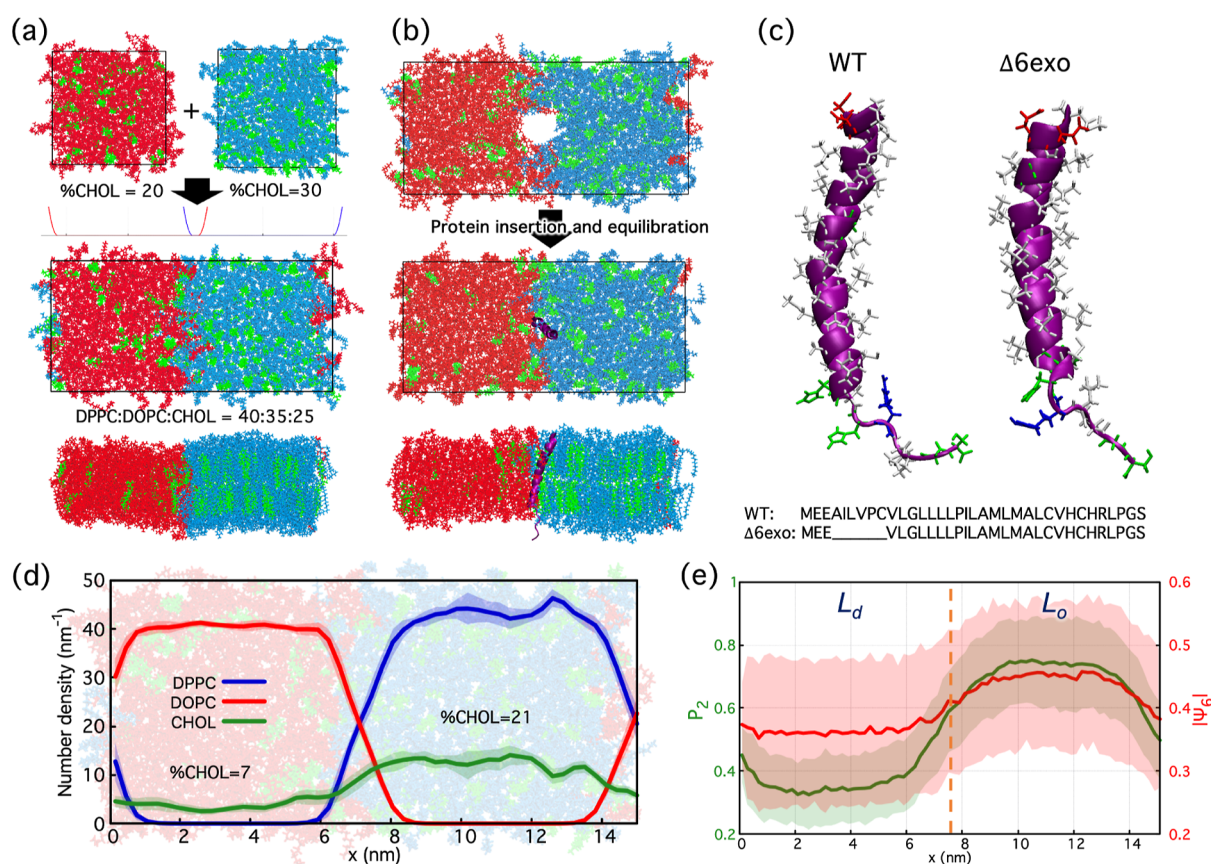


Figure 1. (a) Schematics depicting the construction of the AA phase-separated membrane from two binary membranes (top) with flat-bottom potential restraints of the top-view (middle) and side-view (bottom). DPPC, DOPC, and CHOL are colored blue, red, and green, respectively. (b) Schematics depicting the protein insertion method. A hole at the interface is generated by applying a repulsive cylindrical potential. The top- and side-view of the final equilibrated protein-inserted membranes are shown in the middle and bottom panels. (c) Structures of wild-type (WT) and truncated ($\Delta 6\text{exo}$) trLAT protein with helical regions are shown with a purple ribbon. Residues with nonpolar, polar, acidic, and basic side chains are represented as white, green, red, and blue, respectively. (d) The number density of DPPC, DOPC, and CHOL is plotted as a function of the x -coordinate after equilibration. The mole fraction of cholesterol is converged to 7 and 21 mol % at the L_d and L_o domain, respectively. (e) P_2 and $|w_6|$ of lipids are plotted as a function of the x -coordinate. Shading is used to represent values within one standard deviation of the mean.

Although CG MD simulations can provide an extensive sampling of protein and lipid conformation, CG protein in the MARTINI model has a preassigned secondary structure, thus relevant free energy changes may not be accurately estimated during protein translation between membrane domains. As such, the use of a CG description of proteins has limitations in capturing significant conformational change, electrostatic interactions, and avoiding protein aggregation.^{31,32} In studies of protein partitioning using MARTINI force fields, proteins known to associate with lipid rafts tend to accumulate at the domain interface in smaller systems and partition to the L_d domains in large systems.^{24,28,33} Taken together, those observations highlight the importance of assessing the partitioning free energy of proteins in AA resolution.

In a previous study, we proposed an efficient simulation methodology using CG MARTINI models of equilibrium partitioning of proteins between the L_d and L_o domains in phase-separated lipid bilayers.²⁸ We demonstrated that in the presence of the appropriate restraining potentials, a smaller system can exhibit stable phase separation with L_d and L_o domains comparable to those observed in larger systems without restraints.²⁸ In this study, we extend this method to simulations of AA models of lipid membranes. Using a phase-separated bilayer with restraining potentials, we calculate the potential of mean force (PMF) of the partitioning of a model

TM protein. We compare the results derived for AA and CG models of identical systems. Energy decomposition is used to identify similarities and differences underlying the free energy landscapes of the AA and CG model systems.

MODEL AND METHODS

AA CHARMM36 Model Simulations. The CHARMM36 force field³⁴ was used to simulate AA ternary lipid bilayer. The system was prepared in two steps: (1) composition of a phase-separated ternary lipid mixture from two binary lipid mixtures using a flat-bottom restraining potential and (2) insertion of protein into the equilibrated bilayer. All simulations were performed using the GROMACS 2021.4 package^{35,36} under NPT conditions. The leapfrog integration method was used with an integration time step of 2 fs. The system was maintained at a constant temperature of 330 K and pressure of 1 bar. Temperature coupling was achieved using the Nosé–Hoover thermostat with a coupling constant of 1.0 ps, while pressure coupling was controlled with the Parrinello–Rahman barostat, using a coupling constant of 5.0 ps and a compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$. This setup ensured stable simulation conditions throughout the trajectory.

To construct a phase-separated ternary lipid bilayer containing di-C16:0PC (1,2-dipalmitoyl-*sn*-glycero-3-phospho-

choline, DPPC), di-C18:1PC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, DOPC), and cholesterol (CHOL) in a 40:35:25 mol % ratio, we first carried out simulations of two binary lipid mixtures: a bilayer comprising 180 lipids of DPPC/CHOL at an 70:30 mol % ratio and a bilayer comprising 240 lipids of DOPC/CHOL at a 80:20 mol % ratio. These two bilayers were later combined to form the desired ternary lipid bilayer. Initial configurations were generated from CHARMM-GUI *Membrane Builder*³⁷ and included the TIP3P water model and 150 mM of NaCl to model physiological conditions. The simulation systems were then minimized and equilibrated following the standard protocols of CHARMM-GUI *Membrane Builder*. After a 500 ns production run, the final configurations of each bilayer were used to construct a phase-separated ternary bilayer.

The equilibrated DPPC/CHOL and DOPC/CHOL bilayer systems had lateral dimensions of 6.9 and 7.1 nm, which represent the liquid-ordered (L_o) and the liquid-disordered (L_d) domain, respectively. The final configurations of each bilayer system were used to construct the initial configuration of a ternary phase-separated bilayer (see Figure 1a) by placing them in parallel along the x -axis. In order to prevent lipid mixing, the flat-bottom restraining potential of the form of $V(X) = \frac{1}{2}k_{fb}(X - X_{fb})^2H(X - X_{fb})$ was applied to atoms in a phosphate group (PO_4) of DPPC and DOPC, where X_{fb} is the half-width of the flat portion of the potential and k_{fb} is the force constant, which was set to 500 kJ mol⁻¹ nm⁻². H is the Heaviside step function and $X = |x_i - x_{ref}|$ is the x -distance between the position of the i th lipid x_i and the reference position x_{ref} , defined to be the center-of-mass (COM) of the L_o region for DPPC or L_d region for DOPC. To determine X_{fb} , we calculated the average width of the L_o domain ($\bar{X}_{L_o}$) and the L_d domain ($\bar{X}_{L_d}$). X_{fb} was set to $\bar{X}_{L_o}/2 + \Delta$ for DPPC and $\bar{X}_{L_d}/2 + \Delta$ for DOPC with $\Delta = 0.4$ nm to avoid additional lateral packing caused by the flat-bottom restraint. The system was equilibrated for 1 μ s under the restraining potentials.

During equilibration, the initial cholesterol concentration in the binary mixtures was set to be consistent with levels observed in prior AA simulations of de novo phase separation of ternary lipid mixtures, with 30 mol % for the L_o and 20 mol % for L_d . The cholesterol concentration was allowed to change during equilibration, leading to changes in the width of each domain. We updated the values of $\bar{X}_{L_o}$ and $\bar{X}_{L_d}$ every 100 ns and observed convergence to $\bar{X}_{L_o} \approx \bar{X}_{L_d} \approx 7.7$ nm. The final converged system dimensions after the equilibration were 15.3 nm $\times$ 7.1 nm $\times$ 9.2 nm and the mole fraction of cholesterol is converged to 7 and 21 mol % at the L_d and L_o domain, respectively. A 300 ns production run was performed to analyze the structural properties of lipid bilayer presented in Figure 1d,e.

A protein was inserted into the pre-equilibrated ternary bilayer using the automated membrane generation in CHARMM-GUI.³⁸ As shown in Figure 1b, the insertion method involves the creation of a hole at the domain interface using a repulsive cylindrical force, followed by the insertion of a protein into the hole. We chose to study the TM domain of the linker for activation of T cells (trLAT), a single-pass membrane protein that is a standard for studies of protein partitioning in model membrane systems. We simulated the WT trLAT (WT) and its truncated mutant ($\Delta 6\text{exo}$) in which six amino acids from the N-terminal exoplasmic region were

deleted in the mutant. The sequences and structures of those proteins are shown in Figure 1c. Currently, there is no available experimentally determined structure of trLAT, thus we obtained the predicted protein configuration from the Robetta Web server.³⁹ Following the protein insertion, the system was equilibrated using the standard CHARMM-GUI equilibration protocols, and an additional 100 ns of NPT equilibration was performed. The final configuration of the AA phase-separated lipid bilayer with trLAT inserted was used to generate initial structures for the free energy calculation by using umbrella sampling (US), described in the later section.

CG MARTINI Model Simulations. MARTINI2.2 force field^{29,30} was used to simulate CG lipid and protein systems. The initial configuration of the protein–lipid systems was generated using *insane.py* script.⁴⁰ The same lipid compositions were used as in the AA simulations: di-C16:0PC (DPPC), di-C18:2/18:2PC (1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine, DIPC), and cholesterol (CHOL) at 40:35:25 mol %. The lipid bilayer was solvated with 12 nonpolarizable MARTINI water particles per lipid, and 10% of water particles were modeled with antifreeze parameters. 150 mM salt concentration was used to model physiological conditions. The NPT simulations used the leapfrog integration method with a 25 fs time step, a semi-isotropic Berendsen barostat with a 2.0 ps coupling time and 3×10^{-4} bar⁻¹ compressibility, and other simulation parameters corresponding to the “new-rr” setting described on the MARTINI Web site. We also used a velocity-rescaling thermostat with a 1.0 ps coupling time. The initial randomly mixed bilayer exhibits phase separation after 5 μ s of equilibration. After phase separation, a 10 μ s production run was performed to analyze membrane structure and dynamics.

CG MD simulations were performed for (1) an unrestrained ternary lipid bilayer without a protein, to explore the effects of temperature and system size, and (2) a restrained ternary lipid bilayer with a single trLAT protein (WT or $\Delta 6\text{exo}$), to calculate the partitioning free energy from the US. For the unrestrained lipid-only systems, we investigated temperatures of 295 and 330 K and the system's x/y aspect ratios of 2:1 to 3:1, while keeping the dimension along the y -axis fixed. For the simulation of protein–lipid bilayer systems, a bilayer of 838 lipids was used with dimensions of 22.6 nm $\times$ 10.5 nm $\times$ 8.4 nm. MARTINI parameters for trLAT were obtained using *martinize.py*. The secondary structures were determined from AA simulations calculating the α -helix propensity (p_α) where p_α of residue i was defined as the probability of residue i having a hydrogen bond between an amide hydrogen and a carbonyl oxygen. If a residue had $p_\alpha \geq 0.5$, its secondary structure in the MARTINI representation was considered to be *helix* (H), otherwise, it was considered to be *coil* (C).

Partitioning Free Energy Calculations Using US. In order to calculate the PMF of trLAT across the L_d/L_o domain interface, US simulations were performed. The reaction coordinate was defined as the distance r between the COM of trLAT and the COM of unsaturated lipids, i.e., DIPC in CG simulations and DOPC in AA simulations. The range of r is from 0 to $L_x/2$ with a window separation of 0.2 nm. The force constant of the harmonic bias potential was set to 1000 kJ mol⁻¹ nm⁻². Initial configurations of each umbrella window were generated by pulling the protein from $r = 0$ to $r = L_x/2$ at a rate of 5×10^{-5} nm/ns. For the CG simulations, 2 μ s production runs were performed for each umbrella window, following 500 ns of equilibration. For the AA simulations, 300 ns production runs were performed for each umbrella window,

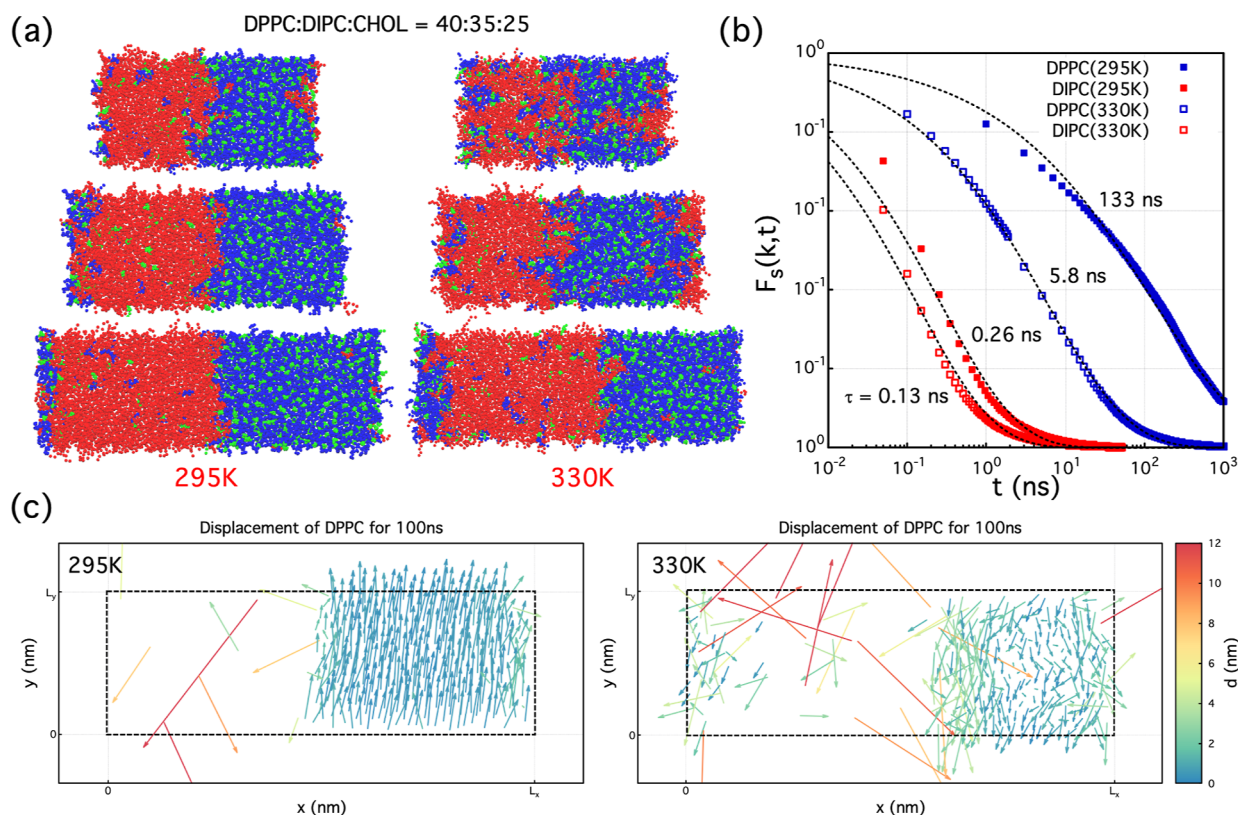


Figure 2. (a) Simulation snapshots of ternary lipid membranes at different system sizes and temperatures obtained from CG simulations. From top to bottom, 900, 1125, and 1350 lipids are simulated for systems with 2:1, 2.5:1, and 3:1 $x:y$ aspect ratios. Blue, red, and green represent DPPC, DIPC, and CHOL, respectively. (b) The self-part of intermediate scattering function ($F_s(k, t)$) of DPPC and DIPC lipids at 295 and 330 K. Black dashed lines indicate the stretched exponential fit. The relaxation times τ extracted from the fitting are shown. (c) The displacement vector map of DPPC in the 3:1 aspect ratio system. The color bar represents the magnitude of a displacement for 100 ns. The dashed box indicates the simulation box.

following 40 ns of equilibration. The temperature of the system was maintained at 330 K during the PMF calculation using both CG and AA force field. The biased probability density obtained from US simulations was unbiased using the weighted histogram analysis method.^{41,42} We have tested the convergence of the PMF by using from 60% to 100% of the entire trajectories from each window (see Figure S1).

Analysis of Membrane Structure and Dynamics. The relative degree of ordering of lipid tails parallel to the bilayer normal was assessed using the liquid crystal order parameter defined

$$P_2 = \frac{1}{2} \langle 3 \cos^2 \theta - 1 \rangle \quad (1)$$

where θ is the angle between the tail vector aligned to the acyl chain and the bilayer normal. For CG lipids (DPPC and DIPC), the tail vector is a bond vector connecting two consecutive tail beads from GL1 to C4A and GL2 to C4B, thus four vectors per tail were counted. For AA lipids (DPPC and DOPC), a tail vector connected every four carbon atoms in the acyl chain to be consistent with CG lipids that employ a 3:1 CG ratio (see Figure S2). The lateral packing of lipids was quantified using the 2D bond-orientational order parameters ψ_6 defined

$$\psi_6^k = \frac{1}{6} \sum_{l \in nn(k)} e^{i6\theta_{kl}} \quad (2)$$

The coordinates of the k th tail particle and its six nearest neighbors ($l \in nn(k)$) were first used to define a plane of best fit and then projected onto that plane. θ_{kl} is the angle between an arbitrary reference vector and a vector connecting k and l in the plane. For CG lipids, C2A, C2B, D2A, and D2B beads were chosen as tail particles for DPPC and DIPC, and the centroid of R1, R2, R3, R4, and R5 was chosen for CHOL. For AA lipids, C28 and C38 were chosen as tail particles for DPPC and DOPC, and the centroid of the steroid ring system was chosen for CHOL. The reference vector was chosen to be the projection of a vector $\vec{r} = (1, 0)$ onto the plane of best fit. The averaged bond orientational order parameter $|\psi_6|$ has a maximum value of 1 when the nearest neighbors present a perfect hexagonal packing. The coordinates of the lipid particles were used to calculate the area per protein (A_p) and lipid (A_{lipids}) using FATS LiM (a fast and robust software to analyze MD simulations of membranes).⁴³ When a protein is included in the system, the coordinate of the residue closest to the coordinates of lipid tails along the z -axis was used.

The structural relaxation of lipid molecules was assessed using the self-part of the intermediate scattering function ($F_s(k, t) = \langle e^{i\vec{k} \cdot (\vec{r}(t) - \vec{r}(0))} \rangle$), where $\vec{r}(t)$ is the position vector of the COM of lipid at time t and $\vec{k}$ is the wave vector. To remove the effect of domain drift, the COM of the lipid is removed from each position vector. The wave vector was set to $k = 2\pi \text{ nm}^{-1}$, corresponding to $|\vec{r}| \approx 1 \text{ nm}$. $F_s(k, t)$ is fitted with a

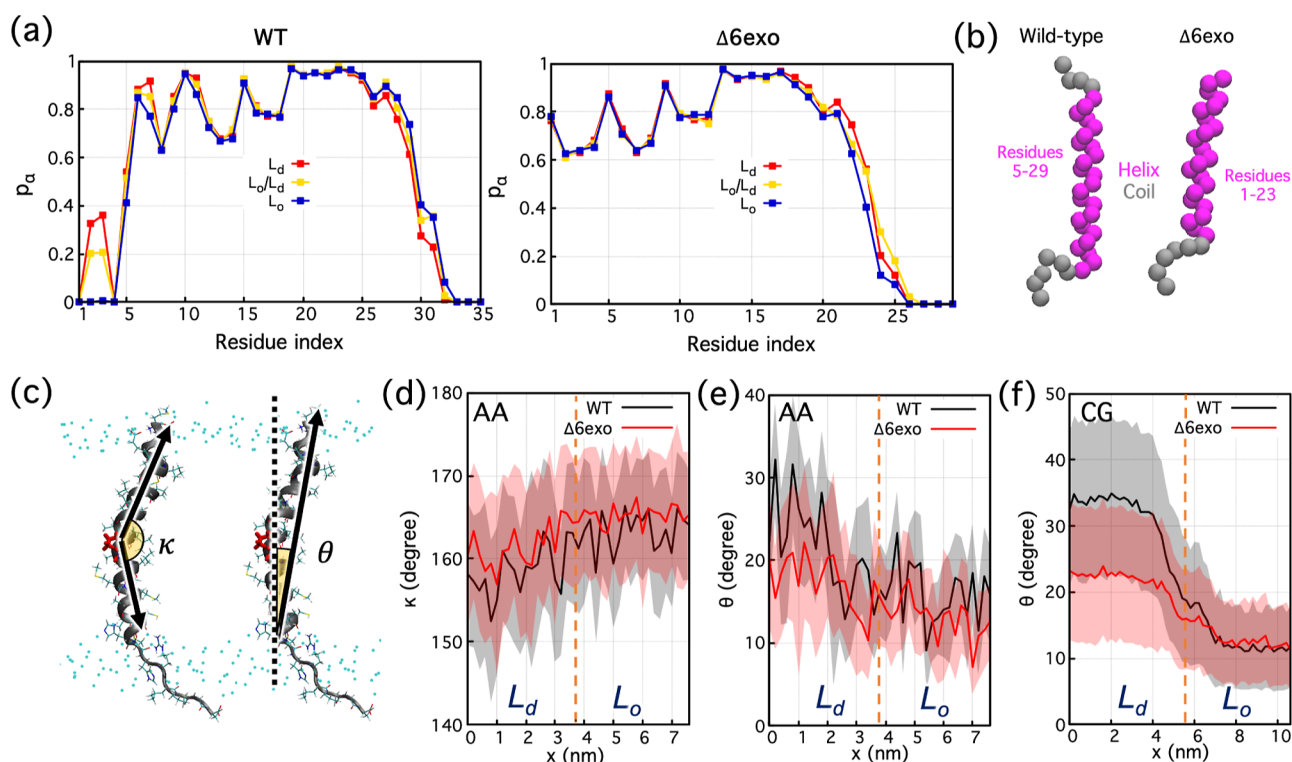


Figure 3. (a) α -Helical propensity (p_α) calculated for residues of WT and $\Delta 6\text{exo}$ trLAT proteins. The helical lengths of WT and $\Delta 6\text{exo}$ are insensitive to the lipid environment. (b) MARTINI representation of WT and $\Delta 6\text{exo}$ trLAT. Colors represent secondary structures where the helix (coil) is purple (gray). (c) Definitions of a hinge (κ) and tilt (θ) angles. Red represents a proline residue of the center of a hinge. The (d) hinge and (e) tilt angles as a function of the x -coordinate of AA trLAT. (f) The tilt angle of trLAT in CG simulations. Shading represents the standard deviation.

stretched-exponential curve, i.e. $F_s(k, t) \approx e^{-(t/\tau)^\beta}$, with two fitting parameters τ and β .

RESULTS AND ANALYSIS

Modeling Phase Separation in a Ternary Lipid Bilayer in the CG Model. In prior work,²⁸ we developed the effective PMF calculations for protein partitioning in CG lipid bilayer systems. In this study, we extend this approach to explore the effects of a restraining potential in a high-temperature membrane composed of a ternary lipid mixture.

Figure 2a shows simulation snapshots of DPPC/DIPC/CHOL membrane systems with varying aspect ratios of a system at 295 and 330 K. The system dimension along the x -direction (L_x) ranged from 22.4 to 33.6 nm while keeping the length along the y -direction constant ($L_y = 11.2$ nm). In 3:1 aspect ratio systems, the membrane displays stable phase separation at both low (295 K) and high (330 K) temperatures, indicating that the macroscopic phase separation temperature is higher than 330 K. In contrast, at a 2:1 aspect ratio, the phase separation is stable at 295 K while the system is mixed at 330 K. The free energy benefit of forming the bulk domains must compensate for the free energy cost of forming the domain interface in the phase-separated state. For larger aspect ratios, the bulk-to-interface ratio is enhanced, relatively stabilizing the phase-separated state. As a result, a phase-separated membrane in AA simulations of the dimensions explored in this study may only be accessible at low temperatures.

We analyzed the lipid dynamics of the 3:1 aspect ratio systems at 295 and 330 K. Figure 2b shows the self-part of an

intermediate scattering function $F_s(k, t)$ for DIPC and DPPC lipids which provides insights into the structural relaxation of lipids. $F_s(k, t)$ is fitted with a stretched-exponential curve, i.e. $F_s(k, t) \approx e^{-(t/\tau)^\beta}$, where τ is a relaxation time scale and β captures the dynamic heterogeneity observed in the lipid diffusion. β is observed to be ~ 0.4 for all systems, reflecting the heterogeneity of lipid diffusion in bulk (fast) and interfacial (slow) regions. The relaxation times τ of each curve are shown in Figure 2b. For DIPC lipids, τ changed from 0.13 to 0.26 ns as the temperature dropped from 330 to 295 K. In contrast, DPPC lipids have τ of 5.8 ns at 330 K increasing to 133 ns at 295 K. Figure 2c depicts the vector map of DPPC lipids where each arrow indicates the displacement vector from $t = 0$ to 100 ns of the COM of individual lipids. The color represents the size of the displacement vector (d). The dynamics at 295 and 330 K are observed to be qualitatively different, as DPPC exhibits collective movements resembling gel-phase dynamics at 295 K but more fluid dynamics at 330 K.

The results suggest that the dynamics of the L_o domain can vary greatly depending on the temperature even though the structural order in the L_o region shows only minor differences at 295 and 330 K (see Figure S2). Systems that exhibit such slow dynamics suffer from less efficient sampling of lipids and protein conformations. This demonstrates the benefit of employing the restraining potential to overcome slow dynamics, as the system will maintain phase-separated states regardless of temperature. This is especially beneficial for AA membrane simulations for which accessible system size and time scales are limited.

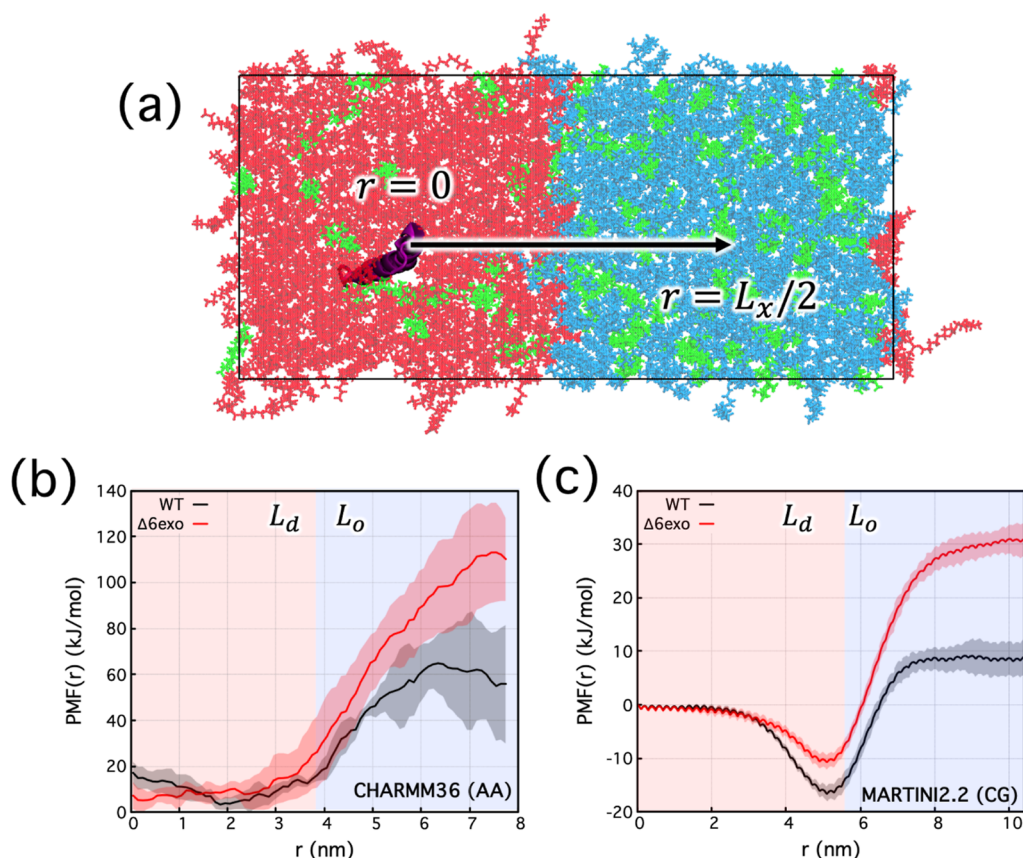


Figure 4. (a) The reaction coordinate (r) used in the US simulations is defined as the distance between the COM of unsaturated lipids (red) and the protein (purple). The potentials of mean force were obtained from (b) AA and (c) CG simulations. Error bars represent the standard deviations estimated using block averages of 100 ns. Red and blue regions indicate the L_d and L_o domains. In both AA and CG simulations, the free energy of the protein is much higher in the L_o domain.

Construction of the AA Model of Phase-Separated Lipid Membranes. We constructed a phase-separated membrane system with AA resolutions (see Figure 1a) using our restraining potential based sampling. We separately equilibrated two binary lipid mixtures for the L_d and L_o patches and integrated them into a single membrane of 420 lipids by imposing a restraining potential. After 1 μs of equilibration at 330 K, the cholesterol concentration converged to 7 and 21 mol % for the L_d and L_o domains, respectively, as shown in Figure 1d. In the absence of the restraining potential, the system will not maintain a phase boundary unless the temperature is reduced below 295 K.

Figure 1e shows the structural order parameters along the x -coordinate measured by P_2 and $|\psi_6|$. The tail ordering and lateral packing are clearly differentiated between the L_o and L_d domains, confirming that our restrained AA membrane represents the characteristics of the disordered and ordered regions of a phase-separated membrane. Despite relatively the small system size ($L_x = 15.5$ nm), the central region of each domain displays the structural characteristics of the bulk domain in terms of P_2 and $|\psi_6|$ order parameters. The values of P_2 and $|\psi_6|$ in the bulk L_o are 0.74 and 0.45, respectively, similar to that observed in simulations of L_o domains.^{27,28}

To introduce a protein into the equilibrated membrane system, we followed an established insertion protocol.³⁸ We generated a weak repulsive cylindrical force centered at the domain interface (see Figure 1b) and then placed a protein in

the hole. Figure 1c shows conformations of the WT and truncated mutant of trLAT after insertion and equilibration.

Dependence of trLAT Protein Structure on Lipid Environment. We analyzed the structure of trLAT as a function of its lipid environments. Figure 3a shows the α -helix propensity along the peptide chain in different membrane domains (L_d , L_o , and L_d/L_o interface). Considering the secondary structure of a residue to be a helix when $p_\alpha \geq 0.5$, a TM helix is observed to extend from residue 5 to 29 for the WT trLAT. In $\Delta 6\text{exo}$, a helix forms from the N-terminus through residue 23. The C-terminal -HRLPGS fragment remains disordered for both WT and $\Delta 6\text{exo}$ due to the presence of charged and polar residues. Importantly, the α -helix propensity for a given peptide shows only a weak dependence on the lipid environment. A notable exception is that WT trLAT presents enhanced helix propensity in the N-terminal region on moving to the L_d domain. This makes trLAT a good model protein system for studies involving protein partitioning as differences in free energy as a function of membrane environment can be assigned to TM helical length and sequence of the integral membrane protein (IMP). This observation also lends support for the use of the MARTINI force field (as shown in Figure 3b) for the study of trLAT.

Figure 3c depicts the hinge angle (κ), with PRO (PRO17 for WT and PRO11 for $\Delta 6\text{exo}$ colored as red), and the tilt angle (θ). Figure 3d,e show κ and θ as a function of the x -coordinate where dashed vertical lines indicate the location of the domain

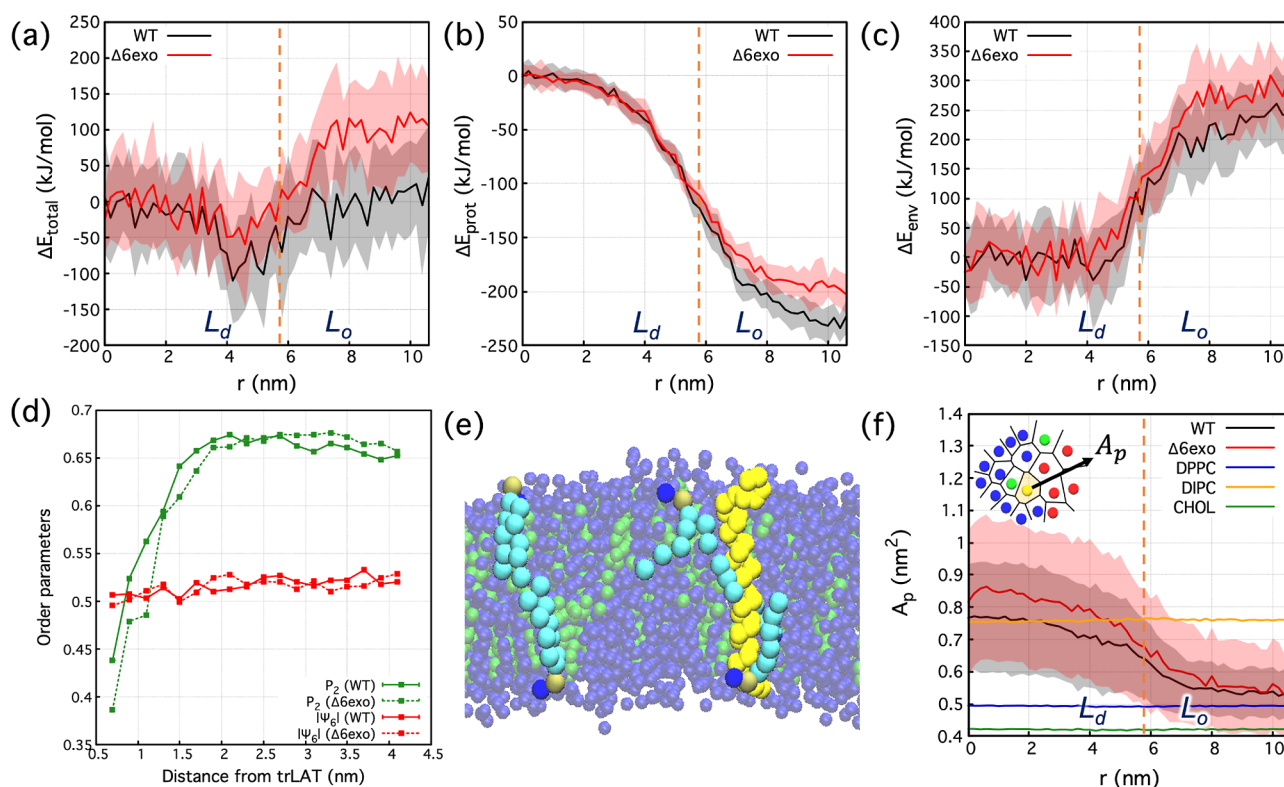


Figure 5. Energy profiles for the protein–lipid system in CG simulations. The energy differences ΔE of (a) the complete system, (b) the protein, and (c) the environment (lipids and solvent) for each umbrella window are plotted as a function of the reaction coordinate r for the CG simulations. Shading indicates the uncertainty obtained from the block average with a block size of 200 ns. (d) P_2 and $|\psi_6|$ order parameters of lipids are plotted as a function of radial distance from trLAT for the protein in the L_o domain. A breakdown of the lipid ordering near the protein is observed. (e) Four DPPC lipids near/far from trLAT (yellow) are highlighted in the L_o domain. Lipid tails near the protein have less ordered conformations. (f) The measured Voronoi tessellation areas of trLAT (A_p) and lipids (A_{lipid}) are plotted as a function of r . Schematic of a Voronoi tessellation of a protein (yellow), DPPC (blue), CHOL (green), and DIPC (red) shown in the inset. The vertical dashed line indicates the location of the interface.

interface. As the L_d domain comprised of DOPC and CHOL is less ordered, membrane thickness is reduced relative to the L_o domain comprised of DPPC and CHOL. As shown in Figure 3d,e, both WT and $\Delta 6\text{exo}$ have higher κ and lower θ angles in the L_o domain (larger x), which we attributed to the observed difference in the membrane thickness. The hinge angle κ is insensitive to the peptide length, while the tilt angle θ is slightly reduced for $\Delta 6\text{exo}$ in the L_d domain due to a shorter helical length.

Figure 3f shows the tilt angle as a function of the x -coordinate in the CG model. The difference in the tilt angle between WT and $\Delta 6\text{exo}$ in the L_d domain is larger than in the AA model as a result of the rigid helix of the CG model that lacks a flexible hinge. As such, while structural changes as a function of the membrane environment are small, those changes are only fully captured by the AA resolution.

Comparison of Free Energy Surfaces of AA and CG Models. We performed US simulations to calculate the PMF for the translation of the COM of trLAT between the L_d and L_o domains in AA and CG models of the phase-separated membrane. The computed PMFs are shown in Figure 4. It has been reported that trLAT tends to preferentially partition into the liquid-disordered (nonraft) domains both in model membrane systems studied using experiments^{19,20} and CG MD simulations.^{24,28} Our results for the free energy of protein partitioning are consistent with previous observations that both WT and $\Delta 6\text{exo}$ trLAT have lower free energy in the L_d

domain. Raft-like L_o domains in model membrane systems used in experiments and simulations lack the compositional and structural diversity of lipid rafts in cells. The high level of ordering of lipids observed in our simulations may be substantially higher than the lipid ordering characteristics of in vivo raft domains. It would overestimate the entropic barrier for a protein to enter the highly ordered L_o .

The computed free energy landscapes for the translation of WT and $\Delta 6\text{exo}$ proteins between the L_d and L_o domains quantify the impact of hydrophobic mismatch on protein partitioning. $\Delta 6\text{exo}$ has a shorter TM helix whose hydrophobic length matches the thickness of the L_d domains. As such, the tilt angle of $\Delta 6\text{exo}$ is smaller than that of WT (see Figure 3e,f). It is reported that in GPMV experiments²³ the raft partition coefficient of $\Delta 6\text{exo}$ is 80% lower compared to that of WT. Our results from both AA and CG simulations show that the free energy difference between the L_d and L_o domain is larger for $\Delta 6\text{exo}$ trLAT, which is consistent with the conjectured role of hydrophobic mismatch in protein partitioning.

CG Model Predicts a Free Energy Minimum at Domain Interface. The most striking difference between the partitioning free energy computed using AA and CG simulations is found at the interface. The computed PMF of AA simulations (Figure 4b) increases gradually as the protein translates from the L_d to L_o domains. In contrast, in CG simulations, a distinct free energy minimum is found at the domain interface. These calculations represent one of the few

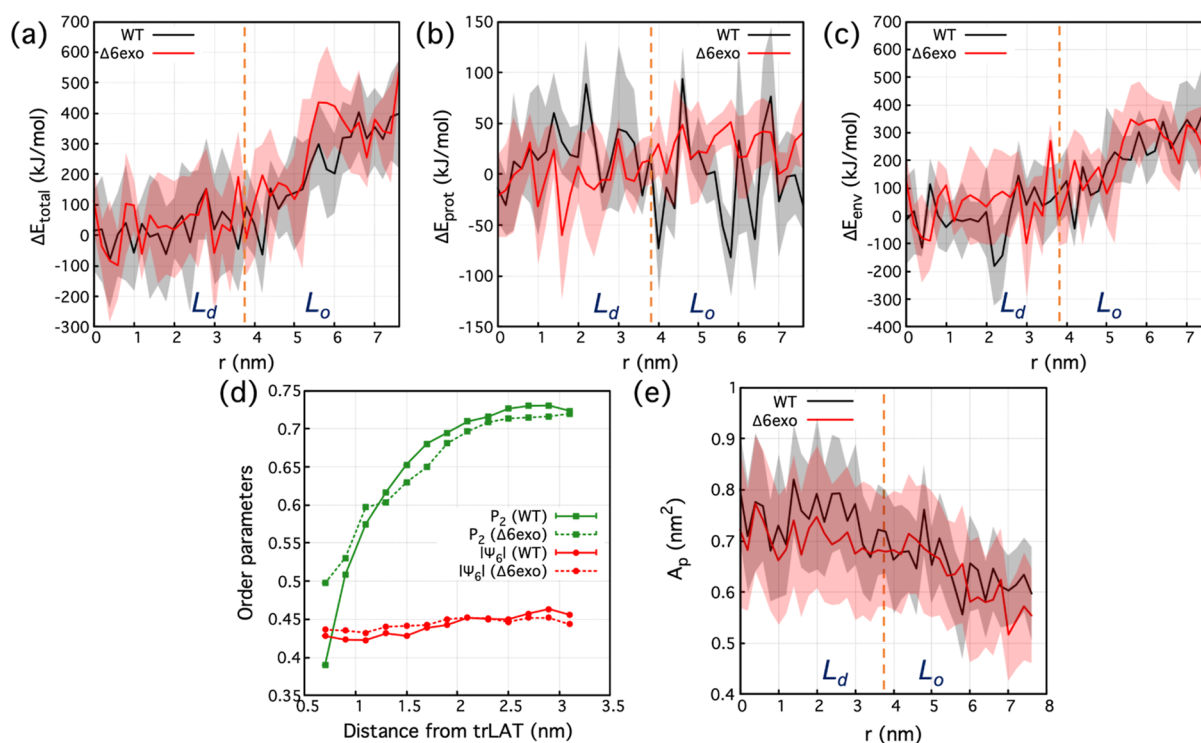


Figure 6. Energy profiles for the protein–lipid system in AA simulations. The energy differences of (a) the entire system, (b) the protein, and (c) the environment (lipids and solvent) at each umbrella window are plotted as a function of the reaction coordinate r in AA simulations. Shading indicates the uncertainty obtained from block averaging with a block size of 200 ns. (d) P_2 and $|\psi_6|$ order parameters of lipids are plotted as a function of the radial distance from trLAT when the protein is in the L_o domain. A breakdown of the lipid ordering near the protein is observed. (e) The Voronoi area of trLAT (A_p) is plotted as a function of r . The vertical dashed line indicates the location of the interface.

studies to quantitatively assess the partitioning free energy in AA simulations. In order to understand the difference between PMFs in AA and CG simulations, we performed an energy decomposition analysis.

Figure 5 depicts ΔE_{total} of CG membrane as a function of r . Figure 5a shows that the total energy of the system (ΔE_{total}) is lowest where PMF of WT and $\Delta 6\text{exo}$ trLAT (Figure 4c) is lowest. In addition, the relative energy difference between the L_o versus L_d domain is larger for the $\Delta 6\text{exo}$ trLAT, indicating that lower affinity for the L_o domain observed in the PMFs can be attributed to the difference in energy.

We decompose the total energy into contributions from a protein and its environment (membrane and solvent) where $\Delta E_{\text{total}} = \Delta E_{\text{prot}} + \Delta E_{\text{env}}$. $\Delta E_{\text{prot}} = \Delta E_{p-p} + \Delta E_{p-m} + \Delta E_{p-s}$ counts the internal energy of proteins, protein–membrane interactions, and protein–solvent interactions. Similarly, $\Delta E_{\text{env}} = \Delta E_{m-m} + \Delta E_{m-s} + \Delta E_{s-s}$ is comprised of intra/intermolecular interactions of lipids in the membrane, membrane–solvent interactions, and solvent–solvent interactions. Figure 5b,c show that ΔE_{prot} and ΔE_{env} are both monotonically changing but have opposite trends with respect to r . A decrease in ΔE_{prot} with increasing r indicates that the partitioning into the L_o domain is energetically favorable for trLAT. However, an increase in ΔE_{env} surpasses this energy stabilization. As a result, the overall energy profile becomes nonmonotonic with a minimum at the interface.

Structural Analysis of Lipid Order across Domains. Additional analysis of ΔE_{prot} and ΔE_{env} reveals that the primary factor contributing to the energetic stabilization is the protein–membrane interaction energy ΔE_{p-m} . The energetic penalty resulting from the surrounding environment is mainly driven

by the membrane–membrane interaction energy ΔE_{m-m} (see Figure S3). In Figure S3, ΔE_{p-m} indicates that both WT and $\Delta 6\text{exo}$ have a favorable interaction energy with lipids. Since the protein undergoes only marginal conformational changes, a change in the internal energy of the protein ΔE_{p-p} is negligible. Figure S3 demonstrates that ΔE_{m-m} increases dramatically with respect to r . When a protein is translated into the L_o domain, it disrupts the lipid ordering and favorable lipid–lipid interactions, leading to a substantial energy cost.

Figure 5d illustrates the changes in the lipid order parameters P_2 and $|\psi_6|$ as a function of the radial distance from trLAT in the xy -plane when the protein is positioned in the center of the L_o domain. It shows changes in the structural order induced by the presence of a protein in the L_o domain. The tail ordering measured by P_2 in the bulk L_o domain is around $P_2 \approx 0.67$ which is reduced up to 40% within 2 nm from both WT and $\Delta 6\text{exo}$ proteins. Figure 5e is a simulation snapshot illustrating that DPPC lipids near the protein have less-ordered tails than those far from it. The diminished tail ordering near the protein leads to a significant increase in lipid interaction energy.

In contrast to P_2 , $|\psi_6|$ remains unaffected with the presence of the protein. We present the area of a protein (or lipids) as a function of r calculated from the Voronoi tessellation in Figure 5f. The Voronoi area of trLAT (A_p) provides insight into the configurational entropy of a protein in the lateral dimension. As shown in Figure 5f, the free area available to the protein decreases as it enters the L_o domain. The observed decrease in A_p can be attributed to the ordering of lipids around the protein in the L_o domain, leading to enhanced protein confinement. Moreover, the difference in the area, $\Delta A_p =$

$A_p(L_d) - A_p(L_o)$, is larger for $\Delta 6\text{exo}$, indicating that the entropic barrier to the L_o domain is higher for the shorter trLAT mutant. These results are consistent with the computed PMF (Figure 4b), which suggests that $\Delta 6\text{exo}$ has a higher free energy barrier to the L_o domains than does the WT protein.

The trends observed in AA simulations and associated free energy landscapes for protein partitioning are different from those derived from CG simulations. The free energy of the AA system increases gradually as the protein is translated from the L_d to L_o domain (see Figure 4). The total energy change (ΔE_{total}) also shows a monotonic increase in contrast to the minimum in ΔE_{total} at the interface derived from CG simulations (see Figure 6a). We previously noted that the nonmonotonic trend in the PMF in CG simulations results from the competing effect of ΔE_{prot} and ΔE_{env} . However, the absence of energetic stabilization of ΔE_{prot} in AA simulations (Figure 6b) indicates that ΔE_{total} is dominated by the increase in ΔE_{env} .

The increase in ΔE_{env} and $\Delta E_{\text{m-m}}$ is observed in AA simulation (see Figure S4), similar to observations derived from CG simulations where the increase in ΔE_{env} is dominated by $\Delta E_{\text{m-m}}$. Consistent with CG simulations, the lipid tail ordering is disrupted in the vicinity of the protein (see Figure 6d), and the area occupied by a protein is decreased as it enters the L_o domains (see Figure 6e).

Figure S4 shows $\Delta E_{\text{p-m}}$ and $\Delta E_{\text{p-s}}$ derived from AA simulations. There exists an energy stabilization by protein–lipid interactions (negative $\Delta E_{\text{p-m}}$) but it is mostly compensated by an increase of the protein–solvent energy $\Delta E_{\text{p-s}}$. Overall, the energetic analysis presented above provides insight into the roles of energy and entropy in the free energy of protein partitioning between L_d and L_o domains in phase separated lipid bilayers.

DISCUSSION

Understanding the partitioning of proteins in model membrane systems is crucial to elucidating the nature of protein–lipid interactions. A significant challenge is the observed inconsistency in the assessment of the partitioning between membrane domains. While the free energy of protein partitioning shows that palmitoylation or longer TM helices in IMPs enhance the affinity for L_o domains, proteins believed to be associated with lipid rafts have been observed to be excluded from L_o domains in model membrane systems.⁴⁴ This discrepancy highlights the need for better theories of how lipid rafts act to recruit IMPs.

Differences between Model Membranes and Lipid Rafts. In contrast to model membranes, lipid rafts in cells are heterogeneous structures composed of mixtures of a variety of lipids and proteins. They are highly dynamic and can change their composition and size depending on factors involving membrane tension, lipid metabolism, and protein–protein interactions. Furthermore, the protein partitioning to lipid rafts in cells is likely to be influenced by additional factors such as membrane curvature and cytoskeleton interactions. Incorporating those factors into models of lipid raft formation may provide a more realistic representation of lipid rafts and provide a predictive model for protein recruitment to raft domains in physiological conditions.

The formation of lipid rafts in cells is a spontaneous process occurring at physiological temperature. However, synthetic and plasma membrane-derived vesicles have a lower phase transition temperature^{45,46} due to a lack of molecular

mechanisms to maintain the phase-separated states at physiological temperatures. Our simulation results reveal that lipid dynamics in the L_o domain of phase-separated membranes is significantly slower and more gel-like when the temperature was reduced from 330 to 295 K. This result implies that the fluid nature of dynamics in the L_o domain may not be observed in smaller systems characterized by a lower phase transition temperature. Therefore, to improve the modeling of lipid rafts, it is important to address the limitation imposed by small model systems. As demonstrated in this study, one potential solution is the use of restraining potentials to overcome the lower phase transition temperature in small systems and better mimic the behaviors of lipid rafts in vivo.

Entropy Disfavors Protein Insertion in L_o Domains of Simple Model Membranes. It has been observed that highly ordered L_o domains in model membranes exclude protein, which in turn affects both the energy and entropy of protein partitioning. Our study demonstrates that the Voronoi area of proteins decreases in the L_o domain in both atomistic and CG simulations. This suggests that it would be entropically unfavorable for a protein to partition into the L_o domain. Furthermore, the presence of a protein disrupts the ordering of lipid tails and favorable lipid–lipid interactions in the L_o domain, resulting in a substantial energy cost indicated by the increase in $\Delta E_{\text{m-m}}$. This increase in energy is not compensated by the modest increase in lipid entropy so there is an overall increase in the system energy. In order to direct a protein into the L_o domain using thermodynamic driving forces, there must be an increase in the favorable protein–lipid interaction in the L_o or a decrease in the degree of lipid ordering in the L_o domains.

Our simulation results found that trLAT has a strong energetic preference for associating with lipids in the L_o domain as evidenced by the negative $\Delta E_{\text{p-m}}$ in Figures S3 and S4. As such, modifying the protein–lipid interaction may not significantly alter the overall trend in the PMF. Alternatively, incorporating various lipid species or cytoskeleton interactions into the membrane model may suppress the hexagonal ordering of lipids observed in our simulations, reducing the energy penalty associated with local ordering. Another possible approach is to introduce defects or inhomogeneities in the system. This can be achieved by introducing mismatches in the size or shape of the lipids or by introducing defects in the bilayer structure.

Comparison with Alternative Models of the L_o Domain. In recent work conducted by Park et al.,⁴⁷ the free energy of partitioning of trLAT between L_o and L_d domains was explored using AA models based on the CHARMM36m force field and US. A flat-bottom restraining potential was used to enforce a predetermined distribution of DSPC/POPC/CHOL in the L_o/L_d domains at low cholesterol (108:56:56/20:174:26) and high cholesterol (86:68:66/24:142:54).

In that study and this work, similar system sizes were used, with 420 lipids for our AA lipid membrane and 440 lipids in this study, which allows for the formation of three solvation shells of lipids around each protein. However, in this study we allowed cholesterol to diffuse between saturated and unsaturated lipids to achieve dynamic equilibrium, forming two binary domains of DPPC/Chol and DIPC/Chol, where the distribution of cholesterol between the L_o and L_d domains was approximately 2:1, most similar to the low cholesterol conditions in the study of Park et al.⁴⁷

Park et al.⁴⁷ observed that trLAT was solvated by unsaturated lipids in the L_o domain. In previous work, we observed a strong free energy bias against trLAT partitioning into L_o domains. Park et al.⁴⁷ speculated that a possible reason for the different conclusions in our studies was the limitation that we did not allow for the presence of unsaturated lipids in the L_o domain. This may certainly be true. Their model of the L_o domain, that incorporates substantial components of unsaturated lipids at both low and high cholesterol, shows significant disorder that is not observed in the more ordered L_o domains in this work.

Energetic Analysis and Convergence in Sampling. In this study, we compared energetic aspects of CG and AA lipid membranes, but a quantitative comparison of the energy reported in our AA lipid membrane models may have limitations. Park et al.⁴⁷ demonstrated that the mean residence time of lipids solvating the protein reaches up to 500 ns in the L_o domain, making it challenging to perform a reliable quantitative analysis of the free energy using US, even with their extensive 4 μ s sampling at each window. Given this observation, the relatively short sampling time in our simulations may contribute to the large observed free energy difference on the order of 100 kJ/mol in our study. The absence of solvating unsaturated lipids in our AA membranes also contributed to the significant energy differences between domains.

Dependence of Partitioning on Domain Composition. When calculating the partitioning free energy, the major difference between computer simulations and in vivo/in vitro membranes is that most simulation systems only contain one or a few proteins. In reality, L_o domains are likely to accommodate a variety of membrane-associated proteins. A single protein simulation can exaggerate the frustration in the L_o domain during the partitioning. In the L_o domains containing multiple proteins, the target protein may interact with other membrane-associated proteins in a way that contributes to favorable interactions.

This is also the lesson derived from the work of Park et al.⁴⁷ Models of the L_o domain incorporating substantial fractions of unsaturated lipids diminish the entropic and enthalpic penalties associated with protein insertion in more ordered models of the L_o composed of saturated lipid and cholesterol alone. This is true for both computational models as well as model membranes in experiments. Experiments assessing protein partitioning between L_d and L_o domains in phase-separated GUVs, formed by ternary lipid mixtures of POPC/PSM/cholesterol in a ratio 2:2:1, observed partitioning solely to L_d domains for variety of proteins considered to prefer the lipid raft environments.⁴⁴ This is likely due to the formation of highly ordered L_o domain structures from the ternary lipid mixtures. The situation differs substantially from protein partitioning to less ordered L_o domains formed from composed of a more complex lipid mixture. As such, computational and experimental studies designed to provide insight into protein partitioning between lipid domains in vivo must be based on an accurate modeling of the lipid composition of the L_o domain.

CONCLUSIONS

The present study utilized US simulations to calculate the PMF for the translation of trLAT proteins between L_d and L_o domains in both AA and CG simulations. Our findings demonstrate that the partitioning of both WT and truncated

trLAT ($\Delta 6_{\text{exo}}$) to the L_d domain is energetically favorable in agreement with earlier studies. The difference in the free energy landscapes for protein partitioning between the L_d and L_o domains is computed using AA and CG models. In CG simulations, we observed an energetic penalty, associated with the breakdown of membrane order, and an energetic gain resulting from favorable protein–lipid interactions, leading to an energy minimum for protein partitioning to the interface. In AA simulations, the energetic penalty associated with the disruption in membrane order dominates the overall increase in the total system energy.

It remains an outstanding challenge to resolve the differences between in vitro experimental studies and simulation results, showing a preference for IMP partitioning to L_d domains, and ex vivo experimental evidence for IMP partitioning to L_o domains. This underscores the importance of exploring more complex models of L_o domains and lipid rafts, in both computational and in vitro experimental studies, as well as lipidation, to fully assess the thermodynamic driving forces that make partitioning of protein into L_o domains thermodynamically favorable.^{47,48}

To obtain a more accurate understanding of protein–lipid interactions in physiological conditions, it is essential to consider the complexity and diversity of the biological system when designing the system and interpreting simulation results. The current study explores the nature of protein partition at equilibrium. However, it is possible that nonequilibrium dynamics plays an essential role in lipid raft formation, through mechanisms such as lipid condensation and wetting of proteins that serve as nuclei for raft formation.

A beautiful recent review explores the role of lipid solvation in determining the structure and activity of IMPs.⁴⁹ As we and others have shown, varying lipid solvation can modulate membrane protein structure,^{50,51} protein–protein association,^{52–54} and membrane protein activity.⁴⁸ Ultimately, it is crucial to consider the complexity and diversity of the cellular environment when interpreting simulation results. The detailed analysis of the free energy landscape of protein partitioning presented in this work provides valuable insights that can inform more complete models of protein recruitment to lipid domains.

Overall, this study provides insight vital to the design of predictive models of protein partitioning in heterogeneous membranes, or both CG and AA models, required the accurate modeling of protein partition between domains in heterogeneous membranes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jctc.4c00881>.

The Supporting Information contains PMF convergence analysis; the distribution of lipid order parameters obtained from unrestrained CG simulations; energy profiles obtained from CG and AA simulations (PDF)

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Notes

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