Computer Simulation of a DPPC Phospholipid Bilayer: Structural Changes as a Function of Molecular Surface Area

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A series of molecular dynamics computer simulations have been carried out on fully hydrated liquid crystalline dipalmitoyl phosphatidylcholine (DPPC) bilayers at constant surface areas corresponding to 59.3, 62.9, 65.5, or 68.1 Å^2/lipid, the range of values suggested by different experiments in different laboratories. Simulated quantities are compared with those from NMR (deuterium order parameters and contribution of molecular tilt to the order parameter), X-ray scattering (*D*-spacings and detailed density profiles), and partial molar volumes. The results strongly support the value of 62.9 Å^2/DPPC recently proposed by Nagle et al. (*Biophys. J.* **1996**, 70, 1419) and demonstrate the feasibility of a combined experimental, and simulation-based approach for determining membrane structure.

1. Introduction

Phospholipid bilayers have long been studied as models of biological membranes. Under physiologically relevant conditions of temperature and hydration, however, they exist in a fluid liquid crystalline (L_α) state characterized by a high degree of disorder, making their detailed structure difficult to determine by standard techniques such as X-ray diffraction. Less detailed structural information is available from a variety of sources: the average orientation (with respect to the bilayer normal or *z*-axis) of the CH-bond vectors at various carbon positions in the fatty acid chain from nuclear magnetic resonance (NMR) order parameters; the average location along the *z*-axis of selectively deuterated carbon atoms from neutron diffraction; and the *z*-dependent electron density profile from X-ray diffraction. The lipid having the greatest body of experimental structural data is dipalmitoyl phosphatidylcholine (DPPC), which consists of two saturated 16-carbon fatty acid chains connected by a glycerol backbone with a zwitterionic headgroup. The focus of this work is on the fully hydrated L_α state of DPPC multilamellar bilayers.

The experimental data noted in the preceding paragraph yield changes in the atomic location or conformation as a function of the *z* position. Information on the structure in the lateral direction (i.e., the plane of the bilayer) is generally determined through a combination of experimental data and model interpretation. For example, the surface area per molecule, A_0, can be calculated by dividing the volume per lipid (which is obtainable from experiment) by the average thickness of the membrane, where the thickness comes from an *interpretation* of the experimental data. A_0 for DPPC has been estimated to be 58−71 Å^2 from various combinations of NMR, neutron, X-ray, and gravimetric measurements.[1] This range is unacceptably large, especially when considering that separate experiments have shown that changing A_0 by only a few percent leads to membrane rupture.[2] The large uncertainty in the dimensions of the pure lipid membrane makes the determination of small differences due to changes in lipid composition or the presence of solutes such as proteins unlikely. The goal of the research described here is to determine A_0 from a series of molecular dynamics (MD) computer simulations, using experimental data as a guide.

Having laid out this goal, we comment on the appropriateness of the tool we chose to use. First, we consider the comparison to experiment, and second, in the following paragraph, we consider the choice of ensemble. Molecular dynamics simulations involve generating the trajectory of a group of molecules through time. In the present case, we follow the configurations of 72 lipid molecules and approximately 2000 water molecules for a little less than a nanosecond. The natural question to ask is Do the limited length and time scales of the computer experiment prevent us from observing the "true" structure and dynamics of the lipid bilayer. As discussed in previous papers, the small patch of membrane used in the simulation cannot support the undulatory vibrations of a macroscopic membrane[3,4] nor does the simulation time scale allow observation of such phenomenon as the lateral diffusion of a lipid or group of lipids.[5] With these limitations understood, however, we can choose to compare well-sampled properties from the simulation with their corresponding experimental observables. Specifically, the electron density profile that will be compared to our simulation results has been obtained in such a way as to remove the effect of undulations,[6] and the NMR experiment which produces the order parameter data is not affected by lateral diffusion.[7,8]

The second point involves the choice of ensemble. Until recently, MD simulations of interfacial systems, including

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lipid bilayers, have been carried out at constant particle number, volume, and energy (NVE, or microcanonical ensemble) or constant number, volume and temperature (NVT, or canonical).[9−11] It is clear that flexibility of the simulation cell is desirable (e.g., to allow a liquid/liquid system to adjust to the presence of an interface or a lipid bilayer to expand or contract when a peptide is added). To this end, a pressure is applied in one or more directions. Two basic classes are becoming common:[12] constant normal pressure but fixed surface area (NPAT or NPAH, where H is the enthaply); constant normal pressure and constant surface tension (NPγT or NPγH). (See ref 12 for a general discussion of these and other ensembles for interfacial systems.) The difficulty in applying NPγ ensembles to lipid bilayers is that an estimate of the surface tension is necessary. A variety of theoretical and practical arguments have been presented on why the applied surface tension should be zero[13−15] or nonzero.[3,16,17] To add insight to this question, for this study we simulate in the NPAT ensemble and calculate the surface tension at each surface area.

The next section describes the simulation protocol. This is followed by results from the lipid bilayer simulations carried out at four different surface areas and an estimate of A_0. We conclude with a discussion of critical assumptions used to obtain surface areas from experiment.

2. Simulation Methodology

The program Chemistry at HARvard Molecular Mechanics[18] was used with the PARM22b4b parameter set.[19] The CHARMM potential contains terms for bond lengths, bond angles, torsional angles, and improper torsional angles. The interactions between nonbonded atoms are described by Coulombic interactions between partial point charges on the atomic centers and a Lennard−Jones (LJ) 6−12 potential. The LJ potential was switched smoothly to zero over the region from 10−12 Å. Although PARM22b4b was developed with a shifting of the coulombic potential to zero at 12 Å, our earlier observations of severe cutoff-related artifacts led us to utilize the Ewald summation technique for the calculation of electrostatics.[20] The real space summation was truncated at 12 Å using $\kappa = 0.230$ Å^{-1}. The reciprocal space summation was truncated at $k_{xx} = k_{yy} = 6$ and $k_{zz} = 9$.

Four NPAT simulations, each of 72 DPPC molecules forming two 6×6 leaflets, were carried out with the lateral dimensions of the simulation cell ($L_x = L_y$) such that the area per molecule was 59.3, 62.9, 65.5, or 68.1 Å^2. Three-dimensional periodic boundary conditions were applied, and the cell length normal to the membrane (L_z) was allowed to adjust during the simulation to maintain a constant normal pressure of 1 atm. The pressure was maintained by a variant of the extended system formalism, the Langevin Piston algorithm, which reduces oscillations in the cell parameters.[21] A mass of 500 amu and a collision frequency of 5 ps^{-1} were used for the pressure piston. The temperature was maintained at 50 °C, well above the main phase transition temperature and identical to the relevant experiments, by means of the Hoover thermostat.[22]

The initial conditions were developed as described previously.[23,24] Briefly, configurations of individual lipids consistent with a Marcelja mean field were generated by Monte Carlo (MC) simulation, with field values adjusted to obtain agreement with experimental order parameters.[25] These configurations were then randomly chosen and placed onto a hexagonal lattice with a phosphate−phosphate distance (through the center of the bilayer) of 36 Å. Random offsets in x, y, and z were chosen from a Gaussian distribution with a zero mean and a variance of 1 Å. The choline conformations from the MC-generated ensemble were then replaced with those obtained from an earlier MD simulation,[20] because their orientations were too often observed to point toward the bilayer interior (the order parameters do not distinguish a positive or negative projection along the bilayer normal). The initial value of L_z was set to 67.2 Å, the most recent experimental estimate of the bilayer repeat distance.[6] For each area, 25 bilayers were generated in this way using different random seeds and the bilayer with order parameters most closely matching the experimental values was chosen. Water molecules were then added by multiply overlaying a previously equilibrated box of water and removing those water molecules which were less than 1.9 Å from a heavy atom of a lipid or a previously placed water or which were further toward the bilayer center than the average of the carbonyl groups. The number of water molecules/lipid, n_W, equaled 26.5, 29.1, 32.0, 34.8 for $A = 59.3$, 62.9, 65.5, and 68.1 Å^2, respectively (n_W must increase with surface area at a fixed height). Final system sizes ranged from 15000−17000 atoms. The resulting configuration for each area was then energy minimized before beginning the dynamics run.

During the simulation, all bonds involving hydrogen were fixed at their equilibrium distances using the SHAKE algorithm.[26] A time step of 2 fs was employed with a modified leap-frog Verlet integration scheme. A neighbor list, used for calculating the LJ potential and the real space portion of the Ewald sum, was kept to 14 Å and was updated every 20 fs. Coordinate sets were saved every 100 fs for subsequent analysis. Simulations were carried out using either four Hewlett-Packard 9000/735 workstations connected by an FDDI ring or eight processors of an IBM SP2. The simulations at 62.9, 65.5, and 68.1 Å^2 were run for 800 ps, and the simulation at 59.3 Å^2 was run for 600 ps. A picosecond of simulation required approximately 5 h on the HP system or 2 h on the IBM system.

3. Results

(a) Equilibration and Dimensions. The equilibration phase of the simulation was deemed complete at 300

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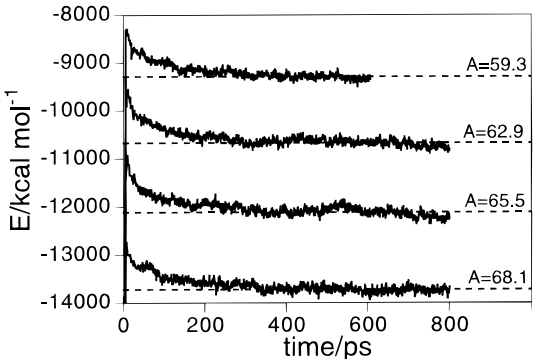

Figure 1. Internal energy as a function of time for the lipid bilayer simulations. Dashed lines show, for each surface area, the average energy calculated from 300 ps to the end of the trajectory. The large differences between the curves is due to the different number of water molecules in each simulation.

ps when the internal energy of the system had stabilized (Figure 1). The coordinates generated after the 300 ps point were used for the analysis presented here. All the simulations produced bilayer structures which were stable on the time scale of the simulation. The average values of L_z, corresponding to the bilayer repeat spacing (D), ranged from 66.8−68.2 Å. Since the D values observed in the simulation depend sensitively on the number of water molecules included, we subtracted the water volume from the average volume of the simulation cell to obtain estimates of the volume per lipid molecule, V_L

$$V_L = \frac{AD}{2} - n_w \times 30.4 \qquad (1)$$

where 30.4 Å^3 is the volume per water molecule obtained from an analysis of the water density in a slab at the center of the water lamella;[27] this molecular volume corresponds to a water density of 0.984 g/mL, very near the experimental value of 0.988 g/ml at 50 °C. The volumes obtained from eq 1 are listed in Table 1; they differ by less than 1% among each other and by at most 1.5% from the experimental value. Thus, the bilayer acts as a largely incompressible fluid and maintains its volume by adjusting the thickness.

(b) Deuterium Order Parameters. The deuterium order parameters, S_{CD}, are obtained experimentally from the quadrapolar splitting, $\Delta\nu_Q$, in an axially averaged line shape,

$$\Delta\nu_Q = \frac{3}{4}\left(\frac{e^2 qQ}{h}\right) S_{CD} \qquad (2)$$

where the term in parenthesis is the quadrapolar coupling constant. From the simulation, S_{CD} is obtained from

$$S_{CD} = \left\langle \frac{3}{2}\cos^2\theta - \frac{1}{2} \right\rangle = \langle P_2(\cos\theta)\rangle \qquad (3)$$

where θ is the angle between the CH-bond vector and the bilayer normal, the brackets denote an average over time and over all the lipids, and axial averaging about the bilayer normal is assumed. The square of the order parameter corresponds to the long time value of the reorientational correlation function, $\langle P_2(\mu(0)\cdot\mu(t))\rangle$, of the bond unit vector, μ. The magnitude of S_{CD}, thus, quantifies the degree of reorientation which occurs on the NMR time scale, i.e., how ordered the molecules are, and their average orientation with respect to the bilayer normal. A vector undergoing isotropic rotation would have an order parameter of zero; typical values of S_{CD} for fluid phase lipid bilayers range from −0.2 at the top of the fatty acid chains to near zero in the terminal methyl groups. Order parameters at various chain positions have been determined experimentally for fully hydrated DPPC at 50 °C.[7,8] Figure 2 compares S_{CD} values from the four simulations with those experimentally obtained for each of the sn-2 chain carbons from C3 to C16. These carbon atoms were chosen because at these positions the protons of each methylene group are equivalent and because experimental data from two laboratories is available for comparison. The order parameters at $A = 59.3$ (Figure 2a) are much higher than those experimentally obtained at all carbon positions, indicating that the lipid molecules are too ordered and that this value of the area is too small. These order parameters were so large that the simulation at $A = 59.3$ was stopped at 600 ps while the others were continued to 800 ps. The agreement with experiment at $A = 62.9$ (Figure 2a) and 65.5 (Figure 2b) is very good, though the $A = 65.5$ results are somewhat higher than those experimentally obtained at the tails of the carbon chains. The shape of the order parameter profile at $A = 68.1$ (Figure 2b) differs from both experiment and the other simulations, with the positions at the middle of the chain having lower order than those experimentally obtained and those at the terminal end having higher order. On the basis of these results, it is clear that $A = 59.3$ is too small a molecular area and that $A = 68.1$ is most likely too large.

A useful order parameter (to be used later in this paper) is $S_{CD}^{plateau}$, which is defined as the average of the CH order parameters in the plateau region, C4−C8. $|S_{CD}^{plateau}|$ is expected to be proportional to the projected length of the lipid along the bilayer normal and, from Table 1, increases as surface area decreases.

(c) Contributions from Internal and Overall Disorder. The increased order at a smaller surface area can result from changes in the internal conformation of the lipid as measured by the chain dihedral distributions (e.g., the gauche/trans ratio) or in the overall molecular orientation (the chain tilt). To separate these two effects, the potential of the mean force for rotation about the C8−C9−C10−C11 dihedral angles was calculated from the angular distributions of each simulation. As shown in Figure 3, the torsional distributions are independent of the molecular area. Evidently, as the surface area is increased, the membrane thins sufficiently to maintain essentially the same environment for the alkyl chains. This is in accord with earlier observations that the bilayer interior is similar to a liquid alkane in terms of chain conformations (but not, clearly, chain tilt).[23] It is counter to the picture of membrane order depending only on the number of gauche defects.

Overall disorder was evaluated by the tilt order parameter, $S_{tilt} = \langle P_2(\cos\theta)\rangle$, of the principal (or long) axis of the moment of inertia tensor. (This procedure relies on two assumptions: (i) that overall rotation is "rigid body" in character and uncoupled from internal motion; (ii) that the eigenvectors of the diffusion tensor are colinear with those of the moment of inertia tensor. Both assumptions have been shown to be very good for rotation of the long axis for liquid alkanes[29] and, thus, are reasonable to make for lipids.) Figure 4 plots the instantaneous S_{tilt} (averaged over lipids) as a function of time for three of the simulations

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Table 1. Summary of Bilayer Structural Parameters

area/Å^2	V_L/Å^3	$-S_{CD}^{plateau}$	S_{tilt}	$\bar{z}_{diff}^{neutron}$/Å	X_{HH}/Å
59.3	1214	0.275	0.74	0.60	40.5
62.9	1219	0.215	0.70	0.76	38.9
65.5	1221	0.210	0.65	1.04	36.9
68.1	1217	0.198	0.64	1.52	35.2
experiment	1232[a]	0.209−0.217[b]	0.69−0.72[c]	N/A	36.4−39.6[d]

[a] Reference 28. [b] References 7 and 8. [c] Reference 8. [d] Reference 6.

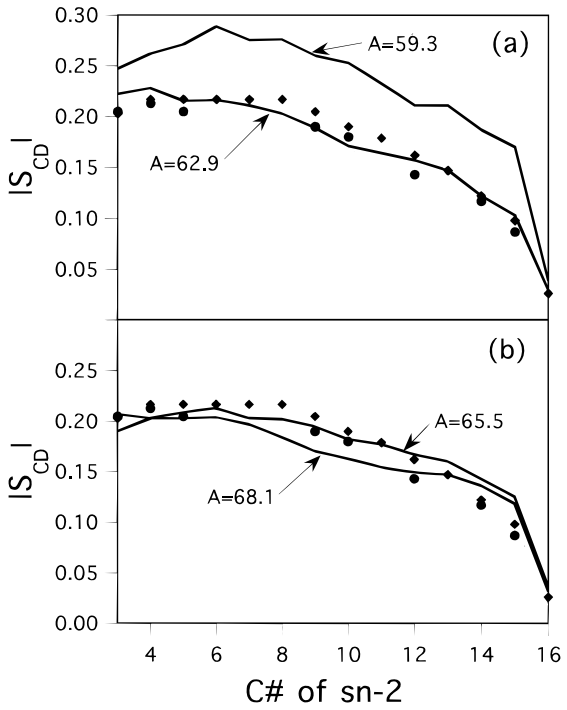

Figure 2. Deuterium order parameters, S_{CD}, for the sn-2 chain of DPPC. (a) 59.3 and 62.9 Å^2; (b) 65.5 and 68.1 Å^2. In both figures the symbols denote experimental values, with circles from Seelig and Seelig[7] and diamonds from Douliez et al.[8] (For certain carbon positions, two values were reported in ref 7 without assignment to the sn-1 or sn-2 chain. We have assigned the value closest to that reported in ref 8 as being the value for the sn-2 chain.)

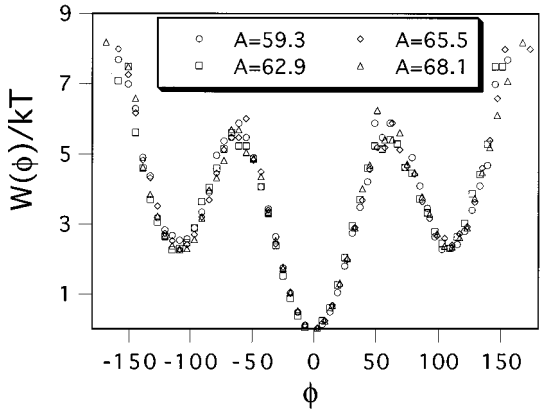

Figure 3. Potential of mean force for rotation about the C8−C9−C10−C11 dihedral angle of the palmitic acid chains.

(for clarity, the results are not displayed for $A = 65.5$ because they were very similar to those at $A = 68.1$). The increasing molecular order with decreasing surface area is seen from the time-averaged values of S_{tilt}: 0.74, 0.70, 0.65, and 0.64 for $A = 59.3$, 62.9, 65.5 and 68.1, respectively; the statistical error is estimated to be ±0.03 from the variance among the 72 lipids. Experimental values of S_{tilt} for dimyristoyl phosphatidylcholine (DMPC) have been obtained by Duforc and co-workers[8] at a variety of

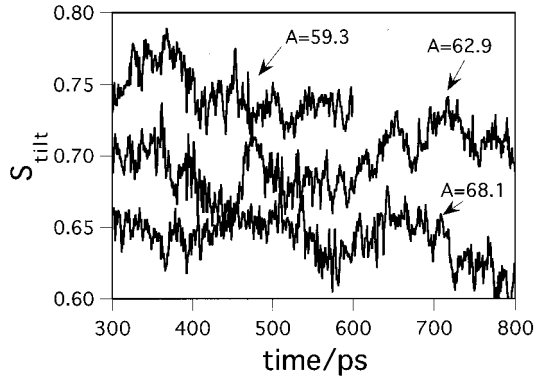

Figure 4. Tilt order parameter, S_{tilt}, calculated from the vector defining the principal axis of each lipid. The upper, middle, and lower lines are from the simulations at 59.3, 62.9, and 68.1 Å^2, respectively.

temperatures. To compare DMPC (which contains 14 carbon fatty acid chains and melts at a lower temperature) with DPPC, we use the range from 0.69 to 0.72, the values obtained for DMPC at the same reduced temperature as the simulations; this indicates that the molecular ordering observed in the $A = 62.9$ simulation most closely matches that observed experimentally.

(d) Density Profiles. We first consider the density profiles from older neutron scattering data on isotopically labeled carbon atoms[30,31] and then from more recent X-ray data.[6]

Table 2 compares the average distance from the bilayer center to the carbon atom of interest from the four simulations and the neutron scattering experiment. Although this data does not provide a stringent test of the simulation accuracy (most points are within an Angstrom of each other), some trends are evident. When the average deviation, $Z_{diff}^{neutron} = |z_{sim} - z_{exp}|$, is calculated over all carbon positions, the values for the simulations at $A =$ 59.3 and 62.9 (0.60 and 0.76 Å, respectively) are well within the stated precision of the experiment, the value of 1.04 Å for $A = 65.5$ is approximately equal to experimental error and the simulation at $A = 68.1$ shows poor agreement with experiment (1.52 Å). We must add two qualifications concerning this comparison: (i) the experiment was carried out at a lower hydration level than the simulations ($D =$ 54.1 Å), although differences in bilayer structure are expected to be small;[6] (ii) discrepancies are unavoidable because the distances between C4 and C5 and between C14 and C15 in the experimental data set exceed the carbon−carbon bond length.

Electron density profiles, displayed in Figure 5, were calculated from the trajectories by dividing the simulation cells into 0.1 Å slabs and determining the time-averaged number of electrons in each slab. The peaks show the position of the electron-rich phosphate section of the

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Table 2. Average Distance (Å) from the Bilayer Center to Various Carbon Positions in the Lipid Bilayer[a]

C position	$A = 59.3$	$A = 62.9$	$A = 65.5$	$A = 68.1$	exp[30,31]
C_γ	21.6 ± 0.3	20.7 ± 0.2	20.0 ± 0.2	19.5 ± 0.2	21.8 ± 0.6
C_α	21.0 ± 0.3	20.0 ± 0.2	19.5 ± 0.2	18.9 ± 0.2	21.2 ± 1.0
C_β	20.6 ± 0.3	19.6 ± 0.2	19.1 ± 0.2	18.5 ± 0.2	21.0 ± 1.0
CG-3	18.2 ± 0.3	17.3 ± 0.2	16.7 ± 0.2	16.0 ± 0.2	17.4 ± 1.5
C4	12.7 ± 0.2	11.9 ± 0.2	11.5 ± 0.2	10.9 ± 0.2	12.2 ± 1.5
C5	11.7 ± 0.2	10.9 ± 0.2	10.5 ± 0.2	9.9 ± 0.2	10.5 ± 1.5
C9	7.6 ± 0.2	7.1 ± 0.2	6.8 ± 0.2	6.2 ± 0.2	8.1 ± 1.0
C14	3.3 ± 0.2	3.1 ± 0.2	3.0 ± 0.2	2.9 ± 0.2	3.6 ± 1.0
C15	2.8 ± 0.2	2.7 ± 0.2	2.6 ± 0.2	2.7 ± 0.2	1.9 ± 1.0

[a] Error estimates for the simulation were determined by dividing the standard deviation of the time-averaged results for the 72 lipids by $\sqrt{72}$. As discussed in ref 32, this is a lower limit to the standard error.

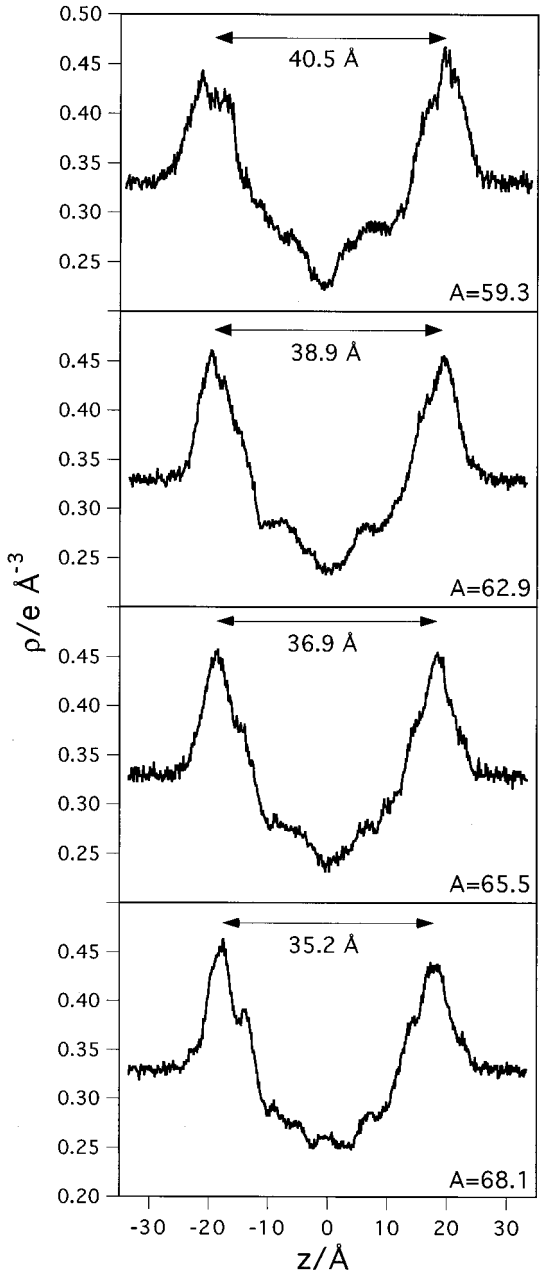

Figure 5. Electron density as a function of z position and peak-to-peak spacing (X_{HH}) for the four simulations. The experimental range for X_{HH} is 36.4−39.6 Å[3] (Nagle et al.[6]). The 95% confidence intervals for the simulated peak positions are approximately that of the phosphate positions, or less than ±1 Å (see discussion in ref 32). Because the plotted densities are not symmetrized with respect to the bilayer center, differences between the two sides also yield a measure of uncertainty in the simulations.

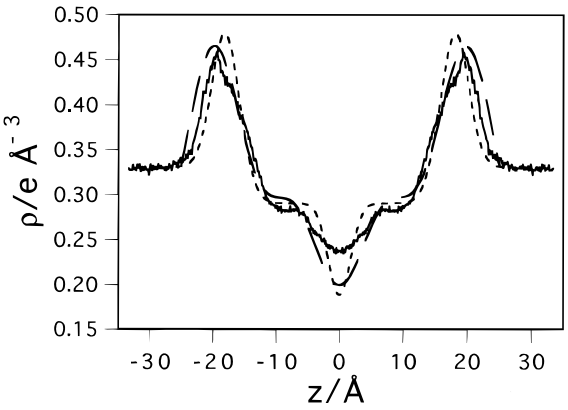

Figure 6. Electron density, now symmetrized with respect to the bilayer center, as a function of z position from the simulation at $A = 62.9$ Å^2/lipid (solid line) and two interpretations of the X-ray diffraction data[6] (dashed lines).

headgroup and the peak-to-peak distance (X_{HH}) is often used to infer the molecular length or changes in length from the experimental data. As evident in Figure 5 and consistent with a constant bilayer volume, peak spacing increases as surface area decreases. Nagle and co-workers[6] recently determined that X_{HH} = 36.4−39.6 Å (the range is associated with differences in data analysis), which brackets the simulated results at $A = 62.9$ and 65.5 but does not bracket those at A = 59.3 and 68.1 (Figure 5). Between the headgroup peaks is a plateau region (occupied primarily by methylene groups) and a trough centered around zero arising from the lower electron density of the terminal methyl groups. The trough at A = 68.1 is wide compared to those at lower surface areas and contains a local maximum at $z = 0$. This can be explained in terms of decreased order leading to more instances of chain upturn and interdigitation. The best agreement of the experimental electron density profile and simulation is found for $A = 62.9$; a comparison is shown on Figure 6. The position of the headgroup peak from the simulation lies between the experimental estimates and the heights of the peak are also in excellent agreement. This is significant because the headgroup peak region is best resolved in the X-ray experiment. For the width of both the methylene plateau and the methyl trough, the simulation results are again midway between the two treatments of the experimental data. Only in the depth of the methyl trough is there a small discrepancy.

Figure 7 shows a snapshot from the trajectory $A = 62.9$. Many of the qualitative structural features described in this section, such as the combination of internal and overall disorder and distributions of atomic groups, are visible in this picture.

(e) Surface Tensions. For our last result, we consider the pressure tensor of the membrane, a quantity which cannot be directly measured experimentally for such a microscopic patch. Anisotropy in the pressure tensor, **P**, leads to a surface tension, γ, which is calculated from the simulation by eq 4. Values of 26, 39, 34, and 35 dyn/cm/

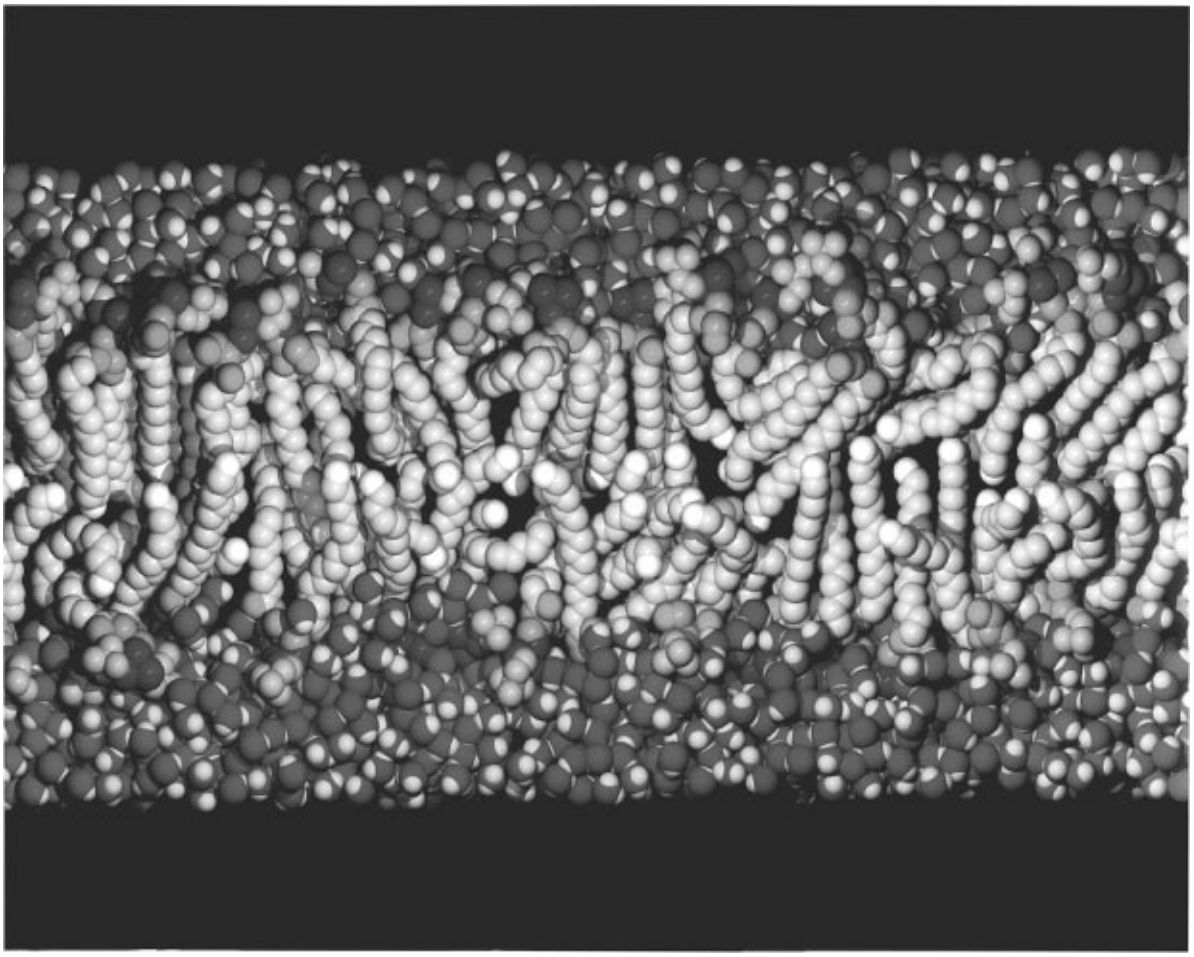

Figure 7. A slab from the 800 ps trajectory frame for $A = 62.9$ Å^2/lipid. Atoms and atom groups are colored as follows: yellow, chain terminal methyl; gray, chain methylene; red, carbonyl and ester oxygen; brown, glycerol carbon; green, phosphate; pink, choline; dark blue, water oxygen; and light blue, water hydrogen. To increase the number of different lipids in the figure while maintaining clarity, a diagonal was taken across three periodic cells and any lipid chain within a predetermined width was included along with its headgroup; sufficient waters were included to duplicate the water/methylene density in the simulation.

$$\gamma = \left\langle L_z \times \left(P_{zz} - \frac{1}{2}(P_{xx} + P_{yy}) \right) \right\rangle \qquad (4)$$

interface were calculated at $A = 59.3$, 62.9, 65.5, and 68.1, respectively. On the basis of recent work (Feller and Pastor, manuscript in preparation), we estimate a standard error in these surface tensions to be about ±8 dyn/cm. Thus, the calculated surface tensions are essentially indistinguishable at the higher areas and only slightly lower at $A = 59.3$. These results imply that the free energy surface for area fluctuations is relatively flat in this range and are consistent with the recent simulations of Tieleman and Berendsen.[15] This is in contrast to our initial NPAH simulations,[33] where calculated surface tensions depended more sensitively on the molecular area when the bilayer was quickly expanded or contracted. The discrepancy is due to including all Coulombic interactions through the use of the Ewald summation, starting each simulation at the prescribed surface area, increasing the run time by an order of magnitude, and, possibly, maintaining a constant temperature for the present simulations. The present results agree with our NPAH studies[33] and our more recent NPAT simulations,[3,20] in that the surface tension is not zero for DPPC when A is in the range of 63 Å^2/lipid. We have argued that the surface tension obtained from the small patch of membrane in the simulation may differ significantly from that of a macroscopic sample due to the confining effect of periodic boundary conditions.[3] The values obtained from these simulations are consistent with our estimates based on a combination of theory and experimental moduli of bending and elasticity.[3,4]

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4. Discussion

Comparison of the available structural information on fully hydrated L_α phase DPPC shows that of the four molecular surface areas per lipid examined in this study, $A = 62.9$ Å^2 most closely reproduces the deuterium and tilt order parameters and the electron density profiles. It is noteworthy that single experiments tended to be consistent with several areas, so that the combination of different experiments *and* simulations at different surface areas were required. This estimate of the surface area is the same as that proposed by Nagle and co-workers[6] from a detailed analysis of their X-ray data.

Over the range of areas studied, the lipid volume was essentially conserved; i.e., changes in area were compensated by changes in membrane thickness. This fluid-like character was also demonstrated by an area independence of isomer populations for the dihedral angles in the middle of the fatty acid chains.

The relative incompressibility of fluid phase lipid bilayers has been exploited by various groups to derive changes in the surface area from changes in the projected length of the lipid (or part of a lipid) along the bilayer normal. Denoting this projection l and assuming constant volume, then $l_1A_1 = l_2A_2$. Hence, measuring Δl yields ΔA,

and if A_1 is known, then A_2 can be determined absolutely. The present set of simulations, where A is known precisely, can be used to test the suitability of particular experimental observables as measures of l. For example, McIntosh and Simon[34] equated changes in X_{HH} with the change in the hydrophobic thickness of the bilayer. To test this assumption, we examine the product of X_{HH} and A from the simulations, which should be constant for their technique to work. From Table 1, the rms deviation among the four calculated volumes is 0.8% of the average. Thus, changes in X_{HH} seem to be an excellent indicator of changes in the molecular length. As a second example, we test the validity of the following relation derived by Nagle[1] to estimate A_0 from deuterium order parameter data,

$$l = \left(\frac{1}{2} - S_{CD}^{\text{plateau}}\right) \times 1.27 \text{ Å}. \quad (5)$$

where l is now the projected length of a CH_2-CH_2 segment, and S_{CD}^{plateau} is the average defined earlier. In practice, the method also requires the volume of two methylene groups, which have been estimated from experiment[28] and simulation,[27] to be 55.2−57.4 Å^3. To test the method, we calculate the volume of two methylene groups for each simulation by multiplying the area per lipid (known precisely) by l (obtained from Table 1 and eq 5). This procedure yields volumes of 58.4, 57.1, 59.1, and 60.4 Å^3 in order of increasing surface area i.e., mostly out of the range 55.2−57.4 Å^3. This discrepancy may be due to not accounting for backtracking of the chains in the derivation of eq 5, which would produce smaller estimates of l and, hence, smaller values of the methylene volume. Interestingly, the volume at $A = 62.9$ Å^2/lipid, 57.1 Å^3, is the lowest of the four values and in best agreement with that experimentally obtained.

In this paper, we have estimated A_0 by comparing simulations at fixed surface areas with experimental data. Our approach is similar to those described in the preceding paragraph in the sense that a model is used to find a value of A_0 consistent with the experimental observations. Aside from our model being more complex, there is the fundamental difference that the simulation is not constrained to give the experimental values in any way. The excellent agreement between the simulation at $A = 62.9$ Å^2 and the available experimental data lends further support to this value and demonstrates the resolving power of atomic level membrane simulations. The *a priori* determination of A_0 from simulation, i.e., locating its value on the free energy surface, remains an elusive and important goal.

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