Fast Lipid Disorientation at the Onset of Membrane Fusion Revealed by Molecular Dynamics Simulations

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ABSTRACT Membrane fusion is a key event in vesicular trafficking in every cell, and many fusion-related proteins have been identified. However, how the actual fusion event occurs has not been elucidated. By using molecular dynamics simulations we found that when even a small region of two membranes is closely apposed such that only a limited number of water molecules remain in the apposed area (e.g., by a fusogenic protein and thermal membrane fluctuations), dramatic lipid disorientation results within 100 ps-2 ns, which might initiate membrane fusion. Up to 12% of phospholipid molecules in the apposing layers had their alkyl chains outside the hydrophobic region, lying almost parallel to the membrane surface or protruding out of the bilayer by 2 ns after two membranes were closely apposed.

INTRODUCTION

Membrane fusion is mediated by fusogenic proteins. However, the main role of the fusogenic proteins is likely to simply facilitate the fusion rather than actively drive it (White, 1992; Chernomordik et al., 1997). For example, hemagglutinin (HA) in the influenza virus envelope, which is the most well-characterized viral fusion protein, first attaches to a target membrane, bringing both target and viral membranes in close proximity, and then, with lowering of the pH (in endosomes), conformational changes of HA further bring the two membranes very close to each other (Wilson et al., 1981; Bullough et al., 1994). The conformational changes induce insertion of the hydrophobic α-helical fusion peptide that had been embedded in the molecule into the target membrane, which leads to membrane fusion without the help of other proteins. An envelope protein of HIV, gp160, as triggered by CD4 binding, also inserts the fusion peptide into the target membrane, which brings the viral and the cellular membranes as close as within 15 Å (Weissenhorn et al., 1997, 1999). Therefore, the role of the fusogenic proteins appears to be to bring two membranes very close to each other. This, perhaps coupled with thermal fluctuations of the membranes, could induce domains where the number of water molecules between the two membranes becomes very small, causing strong interactions between the apposing layers. These interactions may induce processes yet unknown which eventually lead to membrane fusion. In intracellular membrane fusion, fusion between a transport vesicle and the target membrane is triggered by vesicle (v)-SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein [NSF] attachment protein [SNAP] receptor) and target membrane (t)-SNAREs. These two proteins are found to assemble into a hairpinlike complex (SNAREpin, Poirier et al., 1998; Sutton et al., 1998). This complex places the membranes in close apposition and induces fusion of two membranes. The transmembrane domains of these proteins are thought to be essential for exerting force to put two membranes closer together (McNew et al., 2000). The function of these proteins may also be to produce membrane domains where the two membranes are apposed so closely that very little water is left in the intermembrane space.

Two models of membrane fusion proceeding through different intermediate structures have been proposed: the inverted micelle intermediate model (Siegel, 1987) and the hemifusion intermediate model (Melikyan et al., 1997) (Fig. 1). Formation of these intermediate structures requires that many lipid alkyl chains lie almost parallel to the original membrane surface or protrude out of the original membrane (Fig. 1, *b* and *d*, molecules colored pink). Experimental examination of the models has turned out to be difficult, because it has not been possible to exclusively detect the signals from the contact areas due to large background signals from the bulk of the bilayer. Accordingly, we performed molecular dynamics (MD) simulations to study the probable molecular events in the contact area of two bilayers. We built three MD systems, each containing two dimyristoylphosphatidylcholine (DMPC) bilayers placed side by side. The constructed systems differ in the number of water molecules in the intervening space between the two bilayers. Experimentally, spontaneous fusion between DMPC membranes proceeds very slowly (Prestegard and Fellmeth, 1974). However, the rate of membrane fusion can be greatly increased under the conditions that force two membranes to appose themselves very closely, e.g., by inclusion of HA in the DMPC vesicles (Kawasaki and Ohnishi, 1992), or with

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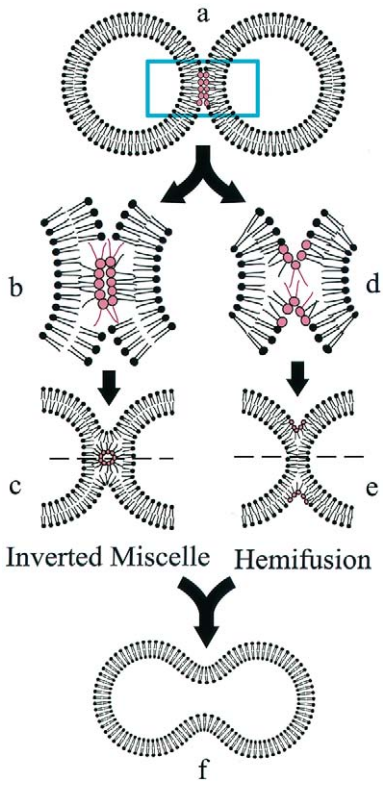

FIGURE 1 Two models for the intermediate stage of the membrane fusion. The contact region of the two apposing membranes (*a*) may form an inverted micelle-like intermediate structure (*b*, *c*) or a hemifusion intermediate structure (*d*, *e*). When the intermediate structures fissure along the dashed line, a fused membrane is generated (*f*).

a surface forces apparatus, application of external force on DMPC bilayers deposited on polymer-cushioned micas (Wong et al., 1999). In this study we addressed the following specific questions: 1) do the headgroups of the apposing lipids dehydrate when two membranes are brought in close proximity?; 2) do lipids change their average conformation and orientation in the membrane?; 3) how closely do two bilayers have to approach each other (how many water molecules can be left between the membranes) before major conformational and orientational changes of their lipid components are promoted?; 4) what are the time scales of such changes?; and furthermore, 5) how do these changes, obtained from the simulation, fit known experimental results?

MATERIALS AND METHODS

Because, in a fully hydrated DMPC bilayer, five water molecules form hydrogen bonds (H-bonds) with each DMPC molecule (Gawrish et al., 1978; Nagle, 1993; Pasenkiewicz-Gierula et al., 1997), we constructed three initial configurations where five (W5), two (W2), and zero (W0) water molecules per DMPC, respectively, were placed between two bilayers (see Fig. 4, *a–c*). To construct an initial configuration, a single bilayer consisting of 56 DMPC molecules (united atom models, 28 in each layer) in the liquid-crystalline state generated previously (the system at 6.0 ns described in Takaoka et al., 2000) was adopted as a starting configuration, and after incorporating on Ewald summation and an external tension (see below), the membrane was further equilibrated. The MD simulation was carried out using AMBER 4.1 (Pearlman et al., 1995) with periodic boundary conditions. The temperature was set at 310 K, which is 14°C above the phase transition temperature of a DMPC bilayer. The Coulombic interactions were calculated using a Ewald summation (Ewald, 1921). van der Waals interactions were cut off at 12 Å. A hardware accelerator for the MD calculation, MD Engine, was used (Toyoda et al., 1999). Based on the Zhang-Nosé algorithm (Zhang et al., 1995), the source program of AMBER was modified to allow the use of the extended ensemble to introduce an external tension of 56 dynes/cm to the system. Good agreement with experimental results was obtained in terms of the order parameter profile (Seelig and Seelig, 1974, Fig. 2); area/lipid (59 $Å^2$ for the simulation, 57–64 $Å^2$ experimentally (Nagle, 1993; Büldt et al., 1979; Petrache et al., 1998)); microscopic MSD (0.6×10^{-6} cm^2/s for the simulation, 1.2×10^{-6} cm^2/s experimentally (Tabony and Perly, 1990)); and the number of *gauche* and *kink* (2.7 and 0.3, respectively, for the simulation; 3.1 and 0.44 (for all gtg's), respectively, experimentally (Tuchtenhagen et al., 1994)). The order parameter obtained by MD simulation for DMPC was compared with the ^{2}H-NMR data obtained by Seelig and Seelig (1974) for DPPC. Because the alkyl chain order parameter depends only on the distance (carbon numbers) from the glycerol group (Hubbell and McConnell, 1971; Kusumi et al., 1986), the data for DMPC can be compared with those of DPPC. Next, two copies of this equilibrated bilayer were placed side-by-side with an average of seven water molecules per DMPC between them (W7). Water molecules were then gradually removed from the space between the apposing layers (Fig. 3 *a*). The systems of apposing membranes containing 5, 2, and 0 water molecules per DMPC were compared among one another at the total run time of 2 ns.

RESULTS

Reorientation of the alkyl chains within 2 ns

To examine conformational and orientational changes of the DMPC molecules in each system, the apposing layers were compared to their isolated sides (for layer nomenclature, see Fig. 3 *b*). Layers 1 and 4 are identical to layers 3 and 2, respectively, in the initial structure of W7. Therefore, the isolated layers serve as a good control for the changes that occur in the apposing layers.

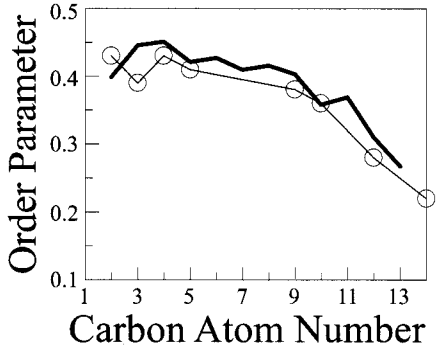

FIGURE 2 The order parameter profile of the simulated DMPC bilayer (*thick line*) as compared with that determined by ^{2}H-NMR (*solid line with open circles*, Seelig and Seelig, 1974). The order parameters in the simulated membrane were calculated using 80-ps trajectories (0.8-ps time step) at the end of the simulation of a single DMPC bilayer.

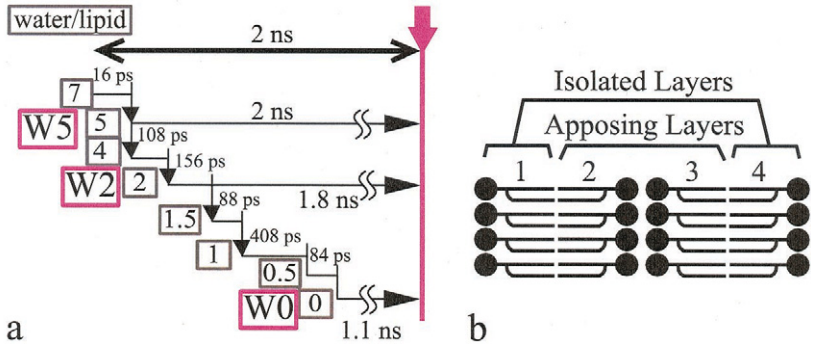

FIGURE 3 (*a*) Diagram showing the process to generate the W5, W2, and W0 systems. Each of these systems was equilibrated for 16 ps at 100 K to eliminate the voids caused by water removal, and then the temperature was raised to 310 K. After the initial states of W5, W2, and W0 were generated, the MD calculation was continued until 2 ns elapsed from the initial state of W7 (including the equilibration periods of intermediate systems). (*b*) Nomenclature of each layer. Layers 2 and 3 are referred to as "apposing layers," while layers 1 and 4 are referred to as "isolated layers."

Fig. 4, *a–c* show the structures of the W5, W2, and W0 systems, respectively, at 2 ns after the start of the simulation. The two apposing membranes maintained their bilayer structures even when they were placed in close proximity in all cases. The boundary between the hydrophobic and the hydrophilic parts of the membrane is defined as the ensemble averaged position of the C_β atoms (the carbon atom located at the center of the glycerol backbone).

The most remarkable change observed in the apposing layers as dehydration of the intervening space proceeds is the emergence of the DMPC molecules whose alkyl chains are located outside the C_β plane lying almost parallel to the membrane surface or protruding from the hydrophobic region (Fig. 4, *a–c*, alkyl chains in the space-filling model with colors other than green). A DMPC molecule in W2 had both alkyl chains (colored cobalt blue, black arrow in Fig. 4 *b*) protruding from the membrane. The methyl-terminal half of the γ-chain of this molecule protruded from the membrane and inserted its tip into the headgroup region of the other apposing layer. The emergence of lipids in such conformations is consistent with both the inverted micelle and the hemifusion intermediate models, described in the Introduction.

Because a single DMPC molecule forms H-bonds to five water molecules, on average, in the presence of excess water, the W5 system represents the state in which all the DMPC molecules in the apposing layers are saturated with water molecules but where no excess water remains in the space between the two apposing layers. Therefore, this result shows that, even if H-bonded water molecules remain between apposing membranes, a dramatic change in the orientation of DMPCs can occur.

Table 1 gives the numbers of alkyl chains that are located outside the C_β plane. In the apposing layers, the number of C14 atoms (terminal methyl groups) found outside the hydrophobic region increased from three to seven as the number of water molecules per DMPC decreased from five to none, while in the isolated layers, the number of C14 atoms found outside the hydrophobic regions remained only one. In other words, up to 12% of the DMPC molecules in the apposing layers had alkyl chains located outside the membrane, whereas <2% did so in the isolated layers. In all but one case, only one of the two alkyl chains of a DMPC molecule protruded outside the membrane (Fig. 4 *c*). In the one case, one of the two alkyl chains repeatedly inserted its end into the hydrophilic domain of the apposing layer (Fig. 4 *b*, cobalt blue, arrow).

It is important to note that while all such changes in the orientation of alkyl chains occurred within 2 ns, some occurred within several tens of picoseconds. When two membranes approach each other, even momentarily as a result of thermal fluctuations, closely enough that there is no bulk water between them, dramatic changes in the orientation of lipid molecules take place almost instantaneously.

Mechanisms of alkyl chain reorientation

Alkyl chains outside the hydrophobic region of the membrane exhibited two types of conformations: type 1 alkyl chains, which lie in parallel with the membrane surface (4 alkyl chains for W0); and type 2, which are bent with their ends protruding outside the C_β plane (4 alkyl chains for W0). Type 1 chains are mainly γ-chains, while type 2 are mainly β-chains. Three of the type 2 β-chains had been bent in the initial configuration with their ends located near the surface of the membrane before initiating the simulation. Three of four chains of type 1 moved out of the membrane during the formation of charge pairs between carbonyl oxygen atoms and choline-methyl groups located near these oxygens (Fig. 5), where a charge pair designates the interaction between positively and negatively charged atoms placed within 4 Å (Creighton, 1993; Pasenkiewicz-Gierula et al., 1999), suggesting that the formation of charge pairs is one of the important mechanisms by which the alkyl chains move out of the membrane.

Fig. 5 shows a typical example. Initially, the purple alkyl chain was embedded deeply in the membrane (Fig. 5 *a*). However, it moved toward the outside of the membrane within 2.6 ns in a step-by-step manner, starting with the proximal portion of the alkyl chain (Fig. 5, *b–g*). Its carbonyl oxygen atom (Oc) and the choline-methyl of a neighboring DMPC (*orange*) are bridged by a water molecule (Fig. 5 *b*, W2). The bridging water molecule then moved

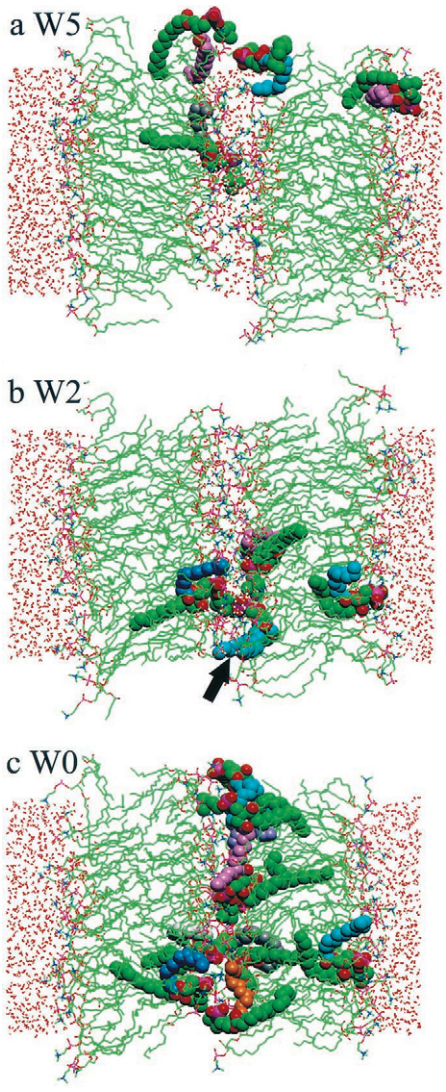

FIGURE 4 Many alkyl chains moved out of the hydrophobic region, lying parallel to the surface or protruding from the hydrophobic region toward the apposing layers. DMPCs having alkyl chains with atoms located outside the hydrophobic/hydrophilic boundary after the total run-time of 2 ns are shown as colored space-filling models; the protruding alkyl chains are shown in colors other than green, while those remaining inside the hydrophobic region are shown in green. (*a*) W5, (*b*) W2, and (*c*) W0. The graphic displays were printed with the *Insight II* molecular modeling system.

out, accompanied by a change of the dihedral angle between the phosphorus atom and the ester oxygen next to the C_α atom from *trans* to *gauche*. This caused the formation of an intramolecular charge pair between the Oc and the choline-methyl group. At the same time, the proximal region of the alkyl chain moved toward the membrane boundary (Fig. 5, *c* and *d*). This alkyl chain movement probably happened because further movement of the choline-methyl group was inhibited by its forming a charge pair with non-ester phosphate oxygen atoms (Op) of the DMPC molecules in the apposing membrane. At 0.86 ns (Fig. 5 *e*, W0), the Oc

TABLE 1 Numbers of DMPC molecules with alkyl chain(s) located outside the average C_β plane

	W5	W2	W0
At C14			
Isolated layers	1	1	1
Apposing layers	3	4	7
At C12			
Isolated layers	0	1	1
Apposing layers	3	4	5
At C10			
Isolated layers	0	1	0
Apposing layers	1	2	2

Each isolated and apposing layer contained 56 DMPC molecules. Numbers of alkyl chains that contained atoms located outside the hydrophobic-hydrophilic boundary after a total simulation time of 2 ns are listed. During the last 200 consecutive time steps (160 ps), carbon atoms (C14, C12, and C10) located outside for more than 100 time steps in total were counted.

formed a charge pair with the choline-methyl group in a neighboring orange-colored molecule and, at the same time, was subjected to repulsion from one of its own Ops (*yellow arrow*). As a result, the alkyl chain swung back and forth (outward, Fig. 5, *e* to *f*, and inward, *f* to *e*), four times, due to repeated *gauche* (Fig. 5 *e*)-*trans* (Fig. 5 *f*) transitions of the dihedral angle between C_γ (C_3) and the oxygen in the ester bond of the γ-chain. The *gauche-trans* transitions were very closely synchronized with the formation (Fig. 5 *e*) and breakage (Fig. 5 *f*) of the charge pair between the Oc and the choline-methyl group of the neighboring DMPC (*orange*). Every time the dihedral angle became *trans*, a greater portion of the alkyl chain moved out of the membrane, until half of the alkyl chain was located outside of the hydrophobic region at 2.6 ns (Fig. 5 *g*). As a result, within 2 ns, the orientation of the alkyl chain changed by ~90° from its initial configuration, showing that large changes in both orientation and conformation can occur within this short period.

Dehydration processes in the lipid headgroup

In the contact area, water molecules that were H-bonded to Ops (and Ocs) of DMPC molecules were frequently replaced by choline-methyl groups via charge pairing. These water molecules moved within the space between apposing layers by continually breaking and reforming H-bonds with neighboring DMPCs. The definition of the H-bond used was Op to H_{water} distance $\leq$ 3.25 Å and the angle of $OpO_{water}H_{water} \leq 35°$, according to Pasenkiewicz-Gierula et al. (1997). Representative movement of a water molecule is shown in Fig. 6. At 124 ps (Fig. 6 *a*), a water molecule (*blue arrow*) was bridging two non-ester phosphate oxygen atoms (Ops) of a DMPC in apposing layers. At 384 ps (Fig. 6 *b*), the water molecule shared by the two DMPC molecules broke free from the DMPC in the right layer (but still keeping the H-bond with the Op of DMPC in the left layer),

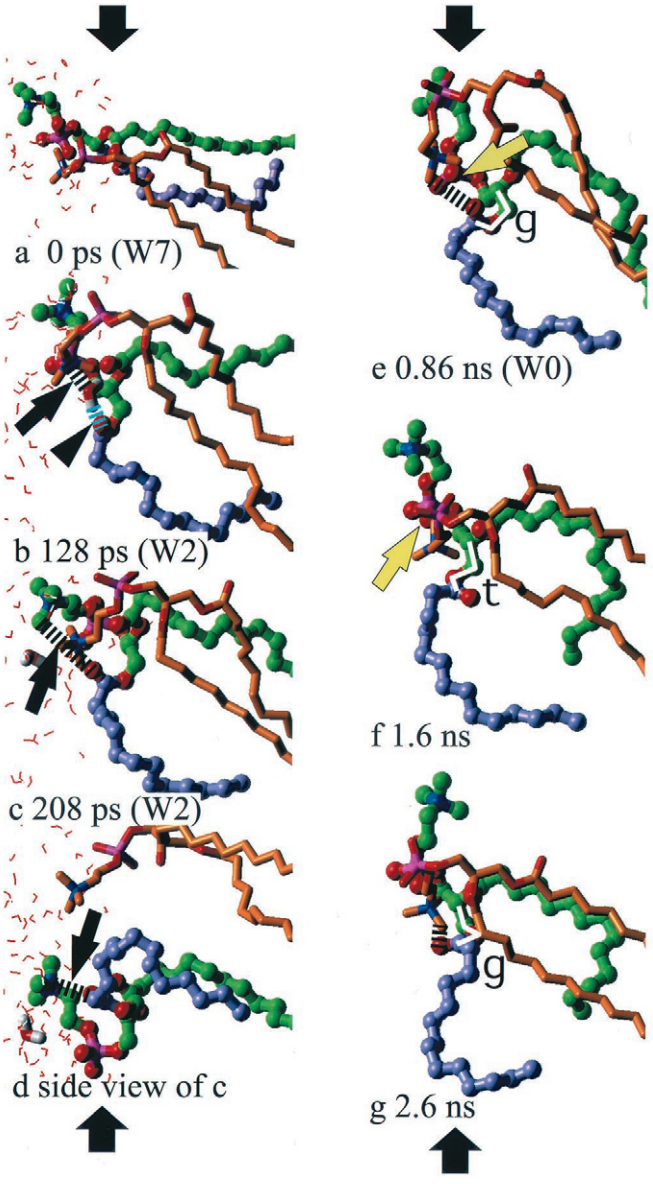

FIGURE 5 A representative case where an alkyl chain moved toward the outside of the membrane, a process coupled with the formation of a charge pair between its carbonyl oxygen atom (Oc) and nearby choline-methyl groups. The purple alkyl chain is the same as the purple chain in W0 in Fig. 4 *c*. The hydrophobic-hydrophilic boundary is shown by the two black arrows placed on the top and bottom of the figure. The black dashed line indicated by a black arrow shows a charge pair (Coulombic interactions between positively and negatively charged atoms located within 4 Å of one another), while the blue dashed line indicated by a black arrowhead shows an H-bond. (*a*) Initially, the purple alkyl chain was deeply embedded in the membrane. (*b*) The Oc and the choline-methyl of a neighboring DMPC (*orange*) are bridged by a water molecule (W2). (*c* and *d*) An intramolecular charge pair between the Oc and the choline-methyl group caused the movement of the proximal region of the alkyl chain toward the membrane boundary. (*e* and *f*) Formation (*e*) and breakage (*f*) of the charge pair between the Oc and the choline-methyl group of the neighboring DMPC (*orange*). (*g*) Half of the alkyl chain was located outside of the hydrophobic region at 2.6 ns. *Gauche* and *trans* conformations are indicated in *e*–*g* by white lines within the molecular models and also by the letters g and t, respectively.

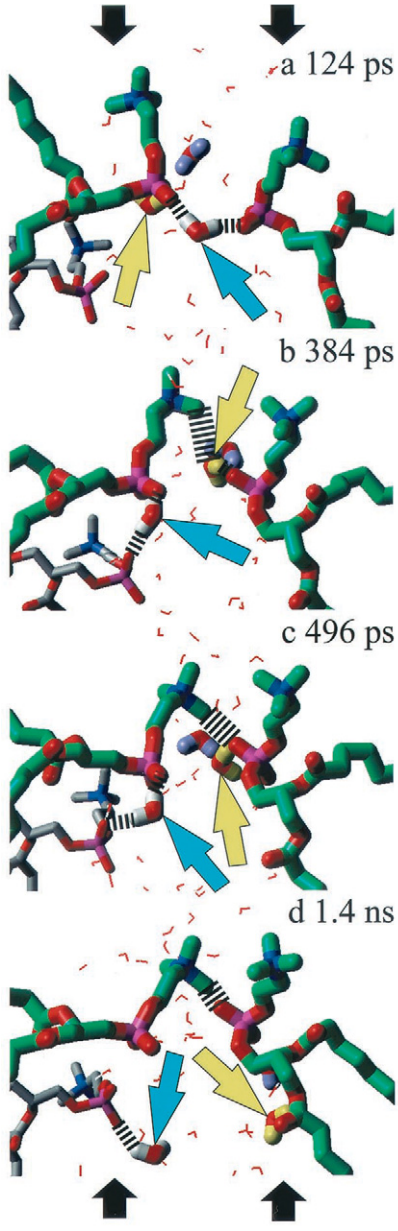

FIGURE 6 A sequence of images that shows typical processes of exchanges between H-bonds and charge pairs in the headgroup region. The approximate position of the hydrophobic and hydrophilic boundary is shown by two pairs of black arrows placed at the top and bottom of the figure. Wide black dashed lines show charge pairs, and narrow dashed lines show H-bonds. (*a*) The initial structure of W2 (124 ps elapsed from initial configuration of W7). Two Ops (*red*) of two DMPC molecules in apposing layers are bridged by H-bonds to a single water molecule (*blue arrow*). (*b*) At 384 ps (260 ps elapsed from *a*), a second water molecule (whose hydrogen atoms are colored *yellow*, *yellow arrow*) came in to bridge the two DMPC molecules, the right DMPC via an H-bond with Op and the left DMPC via a charge pairing with one of the choline-methyl groups. (*c*) At 496 ps (372 ps elapsed from *a*), with the migration of the second water (*yellow arrow*), the Op of the DMPC in the right layer formed a direct charge pair with a choline-methyl group of the DMPC in the left layer. (*d*) At 1.4 ns (1252 ps elapsed from *a*), the second water (*yellow arrow*) moved away, and the first water (*blue arrow*) broke off from the Op that it had initially been H-bonded to in the left layer.

which may have been driven by the changes of the dihedral angle between the ethyl carbon 2 atom of the choline and the oxygen atom next to the phosphorus atom in the left DMPC, the changes of the dihedral angle between the phosphorus atom and the ester oxygen atom next to the C_α in the right DMPC, and the changes of the dihedral angle between the ester oxygen atom and the C_α in the right DMPC. The first water molecule also formed a new H-bond with an Op of a neighboring DMPC (*gray stick model in the left layer*). A second water molecule (whose hydrogen atoms are colored yellow, *yellow arrow*) came in to bridge the two DMPC molecules, the right DMPC via an H-bond with Op and the left DMPC via a charge pairing with one of the choline-methyl groups. Such a conformation had previously been formed among another water molecule (whose hydrogen atoms are colored purple), the choline-methyl of left DMPC and an Op of the right DMPC, 32 ps before. At 496 ps (Fig. 6 *c*), with the migration of the second water (*yellow arrow*), the Op of the DMPC in the right layer formed a direct charge pair with a choline-methyl group of the DMPC in the left layer. This charge pair lasted for >1 ns. The water molecule (*yellow*) that had been bridging these DMPC molecules first broke off from the choline-methyl group, and then 80 ps later from the Op. It migrated by continually forming and breaking H-bonds with different partners, a process that is characteristic of water molecules located in the intervening space (Fig. 6 *d*).

The number of charge pairs per DMPC formed in the apposing layers was 3.6, 4.1, and 6.2 in the W5, W2, and W0 systems, respectively (compare to 1.9 in the isolated layers), which compensated for the decrease in the number of H-bonded water molecules (5/lipid in the fully hydrated system). Tables 2 and 3 shows the frequency of the exchange between H-bonds and charge pairs, and their lifetimes before the exchange. In the apposing layers of the W2 and W5 systems, H-bonds and charge pairs exchange on a fast 100–200 ps time scale, which is in agreement with our previous finding (Pasenkiewicz-Gierula et al., 1999).

TABLE 2 Frequency of exchanges between hydrogen bonds (HB) ($DMPC^{-1}\ ns^{-1}$) and charge pairs (CP) ($DMPC^{-1}\ ns^{-1}$)

	W5	W2	W0
HB to HB			
Isolated layers	89	94	93
Apposing layers	42	5	
HB to CP			
Isolated layers	9	10	11
Apposing layers	17	8	
CP to HB			
Isolated layers	4	4	5
Apposing layers	29	7	
CP to CP			
Isolated layers	18	21	21
Apposing layers	24	24	27

For W5 and W2, 1600-ps trajectories (20-ps time step), and for W0, a 1280-ps trajectory were examined. H-bonds and charge pairs that lasted ≥40 ps were counted. Temporal breakages for <40 ps were neglected.

TABLE 3 Lifetime of hydrogen bonds and charge pairs (ps)

	W5	W2	W0
HB			
Isolated layers	40	39	38
Apposing layers	72	196	
CP			
Isolated layers	79	73	75
Apposing layers (within each layer)	103	141	169
Apposing layers (between two layers)	84	111	156

See Table 2 footnote for details.

We started the simulation with the two bilayers already in close proximity and included only a small number of water molecules in the intervening space, mimicking the condition in which conformational changes of a fusogenic protein (and probably thermal fluctuation of the membranes) bring the two membranes together. The water molecules in the confined space were shown to migrate by continually forming and breaking H-bonds with charged groups of DMPC, which would allow them to move out of the contact area. This is necessary for dehydration to occur when the two membranes are in close contact.

The results of MD simulations may be influenced by the force fields used. However, since both microscopic MSD and order parameters in the isolated layers agree well with experimental results, disorientations of alkyl chains in the apposing layers are not likely due to the force field used here. To examine whether the force field used here leads to fast exchanges between hydrogen bonds and charge pairs, we calculated the hydrogen bond energy between a water molecule and an Op atom and the binding energy of a charge pair (Coulomb + van der Waals) between an Op atom and a choline-methyl, using both the OPLS force field (which is used here) and the CFF91 force field (used in the *Discover* program, MSI, San Diego, CA 1991). The difference between these force fields was <20% for both H-bond and charge pairing energies, suggesting that the exchange between the hydrogen bond and charge pair is not more likely to occur in our system than in systems using other force fields.

DISCUSSION

By using MD simulations we examined the changes in the orientation, conformation, and interaction of lipid molecules when two membranes form a region of a very close contact. Because water molecules hop from one charged group of a DMPC to another an average of every 1 to 2 hundreds of picoseconds, they are likely to readily migrate out of the contact region, with concomitant formation of charge pairs between DMPCs. Within 2 ns after the contact was formed, many alkyl chains that had previously been more or less

parallel to the membrane normal in the hydrophobic interior moved toward the outside, lying parallel to the membrane surface near the headgroup region of the bilayer or protruding from the membrane. Such fast disorientation of lipids is consistent with both inverted micelle and hemifusion intermediate models, which suggests the presence of lipids lying nearly parallel to the original membrane surface or protruding from the membrane in the contact area.

In the experiment of fusion between the red blood cell ghost and cultured cells transfected with HA cDNA, membrane fusion was promoted when oleic acid, which tends to assume cone shapes, was incorporated into the exoplasmic leaflet (apposing layers in this paper), and was inhibited when lysophosphatidylcholines, which have a tendency to assume inverted cone shapes, were incorporated (Chernomordik et al., 1997). This result suggests that fusion is promoted by the inverted micellar or hemifusion structure. In the experiment of fusion among DOPC vesicles induced by gramicidin A (GA) (GA to DOPC molar ratio = 0.1), P^{31}-NMR indicated the presence of the inverted micellar structure in the intermediate state of fusion (Tournois et al., 1990). Several experiments indicated the existence of defects in molecular packing in the area where membrane fusion takes place (Helm et al., 1989, 1992; Monck and Fernandez, 1994; Schmidt et al., 1999). These experimental data are consistent with the simulation results reported here in that many phospholipid alkyl chains in the closely apposed domains protrude from the membrane or lie nearly parallel to the original membrane. Such conformations are likely to make the contacting domain surface more hydrophobic, enhancing the release of hydrating water from the membrane surface.

We believe that the emergence of alkyl chains lying nearly parallel to the original membrane surface or protruding from the membrane in the contact area represents the initiation of membrane fusion, and that the results obtained here support the idea that the role of fusogenic proteins is to bring two membranes sufficiently close to each other so that membrane fusion can be initiated in contact areas where fewer than five water molecules/DMPC exist.

Experimentally, a "flickering" process in which the fusion pore repetitively opens (aqueous phase connects) and closes (aqueous phase separates) was found in measuring the time dependence of the conductance by patch-clamping. In this experiment the flickering was observed as the frequent conductance spikes with lifetimes within 1 ms (Nanavati et al., 1992). In contrast, we propose that the disorientation of lipid molecules can occur at the initial stages of membrane fusion, possibly within the time scale of 2 ns, based on our simulations.

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