Investigations of Ion Channel Structure-Function Relationships Using Molecular Modeling and Experimental Biochemistry

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Chapter 5: Effects of Lipid Composition and Transmembrane Kinking on the Mechanosensitive Channel of Large Conductance (MscL)

Background

Mechanosensitive channels are proposed to play a central role in a variety of physiological processes, including touch, hearing, and circulation (Hamill and Martinac, 2001). The best studied channel of this class is the bacterial mechanosensitive channel of large conductance (MscL) (Batiza et al., 1999; Spencer et al., 1999). Rees and co-workers solved a crystal structure of *M. tuberculosis* MscL (Tb-MscL), showing the channel assembles as a homopentamer (Chang et al., 1998). In the structure, each of the channel subunits contains two transmembrane domains connected by an extracellular loop and a sizable C-terminal domain forming a helical bundle. The first transmembrane domain (TM1) lines the pore, while the second transmembrane domain (TM2) faces the lipid.

MscL is gated by the application of tension to the lipid bilayer (Sukharev et al., 1999), and its gating is not dependent on any other protein or cellular structure as it is fully functional when purified and reconstituted alone in lipid vesicles (Häse et al., 1995). When a bacterium experiences osmotic downshock it swells, producing a tension in the membrane. At a critical value for the tension, MscL opens, acting as a "safety valve" to prevent lysis (Levina et al., 1999; Nakamaru et al., 1999; Wood, 1999). The open channel is not ion selective and can pass relatively large organic ions (Cruickshank et al., 1997), leading to a predicted pore diameter of \approx 40 Å. Researchers have debated whether MscL can pass small proteins, such as thioredoxin and EF-Tu (Ajouz et al., 1998; Berrier et al., 2000; Vasquez-Laslop et al., 2001).

It is clear that interactions between the channel and surrounding lipid are central to MscL gating. Not surprisingly, MscL gating properties are dependent on membrane

lipid composition. Recent work has shown that *E. coli* (Ec) MscL incorporated into lipid vesicles gates at a lower tension when the lipid tails are shortened (Kloda and Martinac, 2001; Perozo et al., 2002b). It is thought that the channel opens more easily because of better hydrophobic matching between the shorter lipid tails of the thinned membrane and the intermediate and/or open states of MscL. Such hydrophobic matching has been proposed to play a major role in channel gating, since the membrane thins upon the application of tension (Hamill and Martinac, 2001; Sukharev et al., 2001b). Other work has shown that the gating physiology of both Tb-MscL (Moe et al., 2000) and Ec-MscL (Sukharev et al., 1993) differs when the channels are expressed in spheroplasts versus incorporated into asolectin vesicles. However, systematic studies of the effects of lipid identity and composition on MscL gating have not been reported.

Molecular dynamics (MD) simulations can give further insight into how lipid composition affects MscL structure and dynamics on the molecular level. As described in Chapter 4, the Tb-MscL crystal structure has provided a starting point for previous MD simulations of the channel embedded in an explicitly represented lipid membrane (Elmore and Dougherty, 2001; Gullingsrud et al., 2001). Such simulations did point to some intriguing protein-lipid interactions (Elmore and Dougherty, 2001). However, each of these previous MD studies considered only one type of lipid, palmitoyloleoylphosphatidylethanolamine (POPE) (Elmore and Dougherty, 2001) or palmitoyloleoylphosphatidylcholine (POPC) (Gullingsrud et al., 2001), so comparisons of the effects of different lipids in directly analogous simulations were not possible. Similarly, most MD studies of membrane proteins only consider a single type of lipid (see (Roux, 2002) and (Forrest and Sansom, 2000) for recent reviews of these

simulations). Recent studies have compared how different lipids affect trajectories of glycophorin (Petrache et al., 2000b) and WALP peptides (Petrache et al., 2002). In particular, Petrache et al. investigated the hydrophobic matching of WALP peptides in membranes with two different tail lengths (2002). As well, other MD studies have focused on characterizing protein interactions with a single type of lipid through interaction energies and changes in lipid properties (Mihailescu and Smith, 2000; Tieleman et al., 1998; Woolf, 1998; Zhu et al., 2001). Using static protein-lipid systems, other computational methods have been used to evaluate the interaction energies between proteins and lipid membranes of varying lipid composition (Arbuzova et al., 2000; Murray et al., 1998; Murray and Honig, 2002). However, these electrostatic studies are limited as they generally do not consider possible changes in protein conformation between different lipid environments.

This chapter describes MD simulations of Tb-MscL embedded in a lipid membrane, addressing the effects of lipid headgroup type and lipid tail length on MscL. In a first set of simulations, directly comparable trajectories of Tb-MscL in POPE and POPC lipid were computed. These simulations show that the structure and dynamics of the MscL channel are directly affected by changes in lipid headgroup, particularly in the C-terminal region. In the second set of simulations, lipid tails were progressively reduced in length, producing a gradual thinning of the membrane over a multinanosecond trajectory. These simulations differ from previous MD simulations of proteins that separately embedded the protein of interest into lipids with differing chain lengths (Petrache et al., 2000b; Petrache et al., 2002). In the present study, gradual thinning could be particularly advantageous, as it may serve as a crude proxy for the

application of tension to the protein-lipid system. Since possible problems in attempting to simulate MscL gating with computationally applied tension have been outlined (Bilston and Mylvaganam, 2002), our indirect approach could be particularly useful. Overall, these shortening simulations support the previously proposed hydrophobic matching of MscL. The lipid shortening simulations implied that gating intermediates might have kinked TM2 helices. Thus, additional simulations and experiments with mutant MscL channels designed to have a TM2 were performed to further consider this possibility. Additionally, in light of recent extensive random mutagenesis of Ec-MscL (Maurer and Dougherty, 2003), consideration of protein-lipid and intersubunit interactions in both sets of trajectories further highlight the central importance of protein-lipid interactions to proper channel function.

Molecular Dynamics Simulations

General Simulation Setup

The Tb-MscL crystal structure (Chang et al., 1998) with all ionizable residues charged (Asp, Glu, Lys, and Arg; no His are present in Tb-MscL) and uncharged N- and C-termini (-NH₂ and -COOH) was used as the starting structure for the channel in all simulations. The termini were left uncharged since they are the extent of the experimentally determined structure but are not the physiological termini of the channel. This structure was initially embedded into an equilibrated POPE membrane (Tieleman and Berendsen, 1998) as in previous Tb-MscL MD simulations (Elmore and Dougherty, 2001). Throughout, the membrane was oriented in the *xy* plane, with the *z*-axis as the membrane normal. The positioning of the channel in the normal of the membrane (the *z*- axis) was consistent with the EPR results of (Perozo et al., 2001). Unlike the simulations in Chapter 4, all residues in the Tb-MscL channel crystal structure (Ala 10-Arg 118) were included in the simulations. This was done because mutagenic data, described in Chapter 3, has begun to probe the functional and structural importance of the Tb-MscL C-terminal region, despite its demonstrated unimportance in Ec-MscL (Blount et al., 1996). Overall, this system included 545 protein residues, 290 POPE lipids, and 23,523 water molecules, for a total of 90,829 atoms; the initial system size is about 9.7 nm x 9.5 nm x 12.4 nm. The full simulation system is shown in Fig. 5.1. This initial system was treated analogously to previous simulations, applying 150 steps of steepest descents minimization to reduce close contacts and gradually heating to 310 K over 20 ps with restraints on all C α atoms of the channel. All subsequent simulations were performed at 310 K.



Figure 5.1: The full simulation system at the end of 100 ps of MD simulation. The channel is shown in white ribbon with residue L81, shown by EPR measurements to lie near the middle of the bilayer (Perozo et al., 2001), highlighted as red spacefilling. Water is depicted as blue wireframe, and lipid molecules are green wireframe with phosphate atoms in yellow spacefilling.

Simulations with Different Lipid Headgroups

In the initial POPE system, the full $C\alpha$ restraints were maintained after heating for 180 ps. Subsequently, they were released in gradual steps over an additional 330 ps; afterwards no restraints were placed on the system.

The headgroup change from POPE to POPC was performed at the 1000 ps frame of the POPE trajectory. This point was chosen because the membrane had been allowed to grossly adjust to the channel after embedding, but the protein structure had not fully entered into its equilibrium interactions with the lipid. At this frame, all the hydrogens attached to the N of the ethanolamine moiety of the POPE headgroups were changed into methyl groups (Fig. 5.2A), and the system was subjected to 50 steps of steepest descents minimization to reduce newly introduced close contacts before extending the POPC trajectory for an additional 6 ns. The POPE trajectory was extended to a total of 5 ns.

Differences between PE and PC headgroup interactions may be due to differences in electrostatics. Recent work has advocated the use of particle mesh Ewald (PME) methods for optimal handling of long-range electrostatics in simulations of lipid bilayers and membrane proteins (Faraldo-Gómez et al., 2002; Feller et al., 1996). However, since others report that the two methods yield quite similar results for some systems (Capener and Sansom, 2002), we compared simulations of Tb-MscL in POPE that utilized either an electrostatic cutoff (1.8 nm) or PME for long-range electrostatics. The channel structures in these simulations were qualitatively similar except for the C-terminal region, which exhibited a greater deviation from the crystal structure in the cutoff simulation. Since recent experimental work, described in Chapter 3, has focused on this region in Tb-MscL and lipid interactions could be important in that region, we opted to use PME (Darden et al., 1993) for long-range electrostatics in POPE and POPC comparison simulations, with 1.0 nm Coulombic and Lennard-Jones cutoffs. The PME calculations utilized a Fourier grid spacing of 0.10 nm and cubic interpolation.



the order in which carbons were removed from lipid tails in lipid

shortening simulations. As discussed in the text, the first two carbons were removed from the oleoyl chain, after which carbons were removed from alternating chains.

Simulations with Lipid Tail Shortening

For lipid shortening simulations, full C α restraints were applied to the heated system for 80 ps. This was followed by a gradual release of those restraints over the following 165 ps of the trajectory; no restraints were placed on the system after that point.

Lipid tail shortening simulations started from the 500 ps frame of a POPE trajectory. Shortening was done by removing the terminal methyl group of one lipid tail and equilibrating the system for 300 ps. After this equilibration time, another methyl was removed, followed by another equilibration window. The order in which carbons were removed is depicted in Fig. 5.2B. Initially, the lipid tails were 16 (palmitovl) and 18

(oleoyl) C atoms in length; this is denoted as 16/18 lipid throughout this paper. The two terminal carbons of the 18 C chain were removed first. After this removal, the lipid was 16/16, and carbons were removed from alternating tails, beginning with the tail that was initially 16 C. In the case where the lipid was shortened to 10/10, the oleoyl double bond was converted to a single bond between the removal of C11 and C12. This was done in order to prevent an unsaturated unit from occurring at the end of a lipid tail, as this could lead to unusual lipid dynamics. After the lipid tails were reduced to the desired length-14/14, 12/12, or 10/10—the trajectory was extended (2 ns for 14/14 and 12/12 simulations, and 5 ns for the 10/10 simulation). Since previous embedded systems began to show equilibration of protein-lipid interactions after 1 ns (Zhu et al., 2001), these extensions allowed the system to adjust before an averaging period for analyses. The 16/18 trajectory was extended to a total of 3 ns.

In order to confirm that the 300 ps equilibration between shortening steps is sufficiently long, an additional shortening trajectory to 14/14 was performed using 600 ps equilibration times. This trajectory was qualitatively similar to the 300 ps step trajectory, although some details of the simulations were different. For example, the 600 ps trajectory showed somewhat less overall bilayer thinning. This might occur because the bilayer initially overcompensates after some lipid shortening events, and this slight overthinning is not fully remediated in the shorter time steps. Also, the relative sizes of the hydrophobic matching effects (border/bulk lipid thickness differences and protein hydrophobic surface length adjustment) differed between the simulations, although their total effect (defined in Eq. 1 below) was the same. Nonetheless, since both methods led to the same interpretations for the system, the more extensive 300 ps step shortening simulations will be discussed in this work.

Lennard-Jones energies and electrostatics were cutoff at 1.0 nm and 1.8 nm, respectively, in these simulations. Similar cutoffs were used in previous simulations of Tb-MscL (Elmore and Dougherty, 2001) and other ion channels, such as KcsA (Shrivastava and Sansom, 2000; Shrivastava et al., 2002). Despite the possible benefits of PME methods described above, cutoffs were utilized for long-range electrostatics in these simulations to increase computational efficiency and allow consideration of a greater variety of lipid lengths and shortening protocols. Moreover, these simulations were primarily aimed at investigating the lipid exposed TM domains, which are generally dominated by hydrophobic interactions, while the main differences between cutoff and PME simulations appeared to be in the C-terminal domain of the channel.

F80P Simulations

The F80P mutation was made to the POPE embedded MscL system described above before minimization. After making the mutation, the system was minimized for 150 steps of steepest descents minimization. Molecular dynamics simulations on the minimized F80P system were performed using the same heating and restraint release protocol utilized for the lipid headgroup simulations. Also, PME methods were used to compute long-range electrostatics for these simulations. The F80P simulation was extended to a total of 5 ns.

Simulation Details

All minimizations and MD simulations were performed using the GROMACS 3 suite of programs (Berendsen et al., 1995; Lindahl et al., 2001). The protocols for MD used in this study were analogous to those used in our previous Tb-MscL simulations (Elmore and Dougherty, 2001). Lipid parameters were from (Berger et al., 1997), with additional parameters for the oleoyl double bond taken from the GROMOS force field. GROMACS atomic parameters were used for protein and water, with some non-bonded parameters determined by standard combination rules. All MD runs used a time step of 2 fs along with the LINCS routine to constrain bond lengths (Hess et al., 1997) and SETTLE to constrain rigid water geometries (Miyamoto and Kollman, 1992). Structures from the trajectories were stored every 0.5 ps for analysis. The NPT ensemble was employed with anisotropic pressure coupling in each direction to 1 bar with a time constant (τ_{n}) of 1.0 ps (Berendsen et al., 1984); this coupling scheme should allow the bilayer system to properly adjust to the embedded protein and alterations in lipid composition. Temperatures were coupled separately for protein, lipid, and solvent to a temperature bath with a coupling constant (τ_t) of 0.1 ps (Berendsen et al., 1984).

Analysis of the trajectories was primarily performed with the GROMACS suite. All average properties, such as energies and distances, were computed over the final 1 ns of a particular trajectory, unless otherwise noted. Interaction energies were averaged over all saved frames, including both short and long-range electrostatics and Lennard-Jones energies. Due to the difficulty of calculating long-range energies between several different portions of the system with PME, per residue long-range interaction energies were calculated with cutoffs of 2.25 nm for simulations run with PME. These energies

were unaffected qualitatively by changing the cutoff value used for this analysis. The definitions of the protein regions—TM1, extracellular loop, TM2 and C-terminal—were residues 15-43, 44-68, 69-89, and 90-118, respectively, as defined previously (Maurer et al., 2000). Hydrogen bonds were defined geometrically as interactions in which the distance between the hydrogen and the acceptor is < 0.25 nm and the interaction angle is $\leq 60^{\circ}$; amide N atoms were omitted as possible hydrogen bond acceptors. Bordering lipids were defined as those with an average minimal distance of 0.35 nm or less from the channel, as in past Tb-MscL simulations (Elmore and Dougherty, 2001). Additional analyses were performed using the HOLE (Smart et al., 1997) and HELANAL (Bansal et al., 2000) programs. Statistical analyses were performed using SPSS (SPSS, Inc.). Molecular graphics were generated with Pymol (DeLano, W.L., http://www.pymol.org).

Simulations with Different Lipid Headgroups

Overall Comments

The transformation of lipid from POPE to POPC resulted in a system that adjusted and stabilized its membrane properties, such as lipid density and lipid P-P distance, in a few ns (data not shown). This included the expected lateral spreading of the PC membrane accompanied by an overall thinning of the bilayer. As well, the protein was generally stable throughout equilibration. The overall channel RMS deviation from the crystal structure was very similar for the first few ns following the change to POPC (Fig. 5.3). However, there was then a rapid transition in the RMS deviation around 2 ns after the alteration. This transition corresponded to changes in the structure of the Cterminal domain. The RMS deviations for other regions of the protein remained very similar between the trajectories and were comparable to those observed in previous Tb-

MscL simulations (Elmore and Dougherty, 2001; Gullingsrud et al., 2001).





The number of protein-lipid hydrogen bonds decreased upon the change from POPE to POPC (Fig. 5.4). Previous simulations in POPE had shown that the lipid ethanolamine moiety was the donor in a high proportion of all MscL-lipid hydrogen bonds (Elmore and Dougherty, 2001). Since such hydrogen bond donation is not possible with POPC, the observed decrease in hydrogen bonding was expected. Diminished hydrogen bonding was most notable in the extracellular loop (Fig. 5.4), which after equilibration had only 40% as many hydrogen bonds in POPC as in POPE. The drop in the C-terminal region was also marked, but of a lower magnitude (69% of POPE).



Lipid-Dependent Conformation of the C-terminal Region

The most notable effects resulting from changing the lipid headgroup from PE to PC are in the Tb-MscL C-terminal region. As discussed above, this region shows a structural transition about 2 ns after the change, resulting in greatly increased RMS deviation from the crystal structure (Fig. 5.3). In this transition, the upper portion of the C-terminal region appears to adjust its conformation, leading the helical regions to approach the membrane more closely (Fig. 5.5). The decrease in C-terminal protein-lipid hydrogen bonding interactions in POPC likely plays a role in this conformational transition. The energetic factors leading to the observed structural changes can be considered by examining protein-lipid interaction energies per residue in the two trajectories (Fig. 5.6A). In POPC, protein-lipid interactions are markedly decreased at Y94 and E102. Without these strong interactions, the channel rearranges in POPC to make interactions at K99 and K100 more favorable. These structural changes bring the C-terminal region closer overall to the membrane (Fig. 5.5B), increasing lipid interaction with D108 and E116 (Fig. 5.6A). As well, these rearrangements lead to the opposite

З

time (ns)

effect in intersubunit interactions at the same sites: decreased intersubunit interactions at K99 and K100 and more favorable intersubunit interactions at E102 (Fig. 5.6B). Most of the residues showing altered lipid interactions are highly conserved among MscL orthologues (Maurer et al., 2000). K99, K100, and E102 are part of a highly charged region found in all orthologues and determined to be essential for channel function in cleavage studies of Ec-MscL (Blount et al., 1996). D108 and E116 also have acidic counterparts in other MscL channels. Thus, lipid interactions noted in these simulations may play a general role in influencing the structure and the functional role of MscL C-terminal regions.

Figure 5.5: Pictures of the final frames of the POPE (A) and POPC (B) trajectories, with the protein shown as white ribbon and the lipid phosphate atoms shown as gray spacefilling.





Figure 5.6: Protein-lipid (A) and intersubunit (B) interaction energies per residue for the C-terminal region in POPE (black) and POPC (gray) simulations.

Thus, it appears that interactions with POPE lipids can promote the crystal structure conformation of the Tb-MscL C-terminal helical domain. Recent data (reported in Chapter 3) has shown that some single site mutations in this region lead to altered thermal stability as determined by CD melting studies. It would be particularly interesting to determine the relationship between these structural changes and Tb-MscL function. As well, the gating tension of Tb-MscL is quite high (compared to that of Ec-

MscL) when expressed in *E. coli* spheroplasts (Moe et al., 2000), which have a high proportion of PE lipid ($\approx 75\%$) in their inner membrane (Raetz, 1978). Conversely, M. tuberculosis membranes contain comparatively little PE lipid, less than 15% of their total lipid (Khuller et al., 1982; Lee et al., 1996). Instead, the majority of their membrane consists of glycolipids, phosphatidylinositol mannosides (PIMs). Although the sugars on PIMs do have hydroxyl groups that could serve as hydrogen bond donors, they would not form interactions as strong as the charged hydrogen bonds from PE headgroups. However, since PIMs are most prevalent in the outer leaflet of the membrane, such interactions may be most relevant in the extracellular loop region discussed below. Interestingly, ornithine-based lipids, which contain an amine moiety and are hypothesized to be functionally interchangable with PE lipids (Wilkinson, 1972), have been isolated from *M. tuberculosis* (Lanéelle et al., 1990); however, there is no direct evidence ornithine lipids are located in the *M. tuberculosis* plasma membrane (Daffé and Draper, 1998). These observations lead to the intriguing idea that the difficulty of gating Tb-MscL in spheroplasts may be related to its adaptation for a different native lipid environment. Such a proposal merits further experimental consideration with electrophysiological and structural measurements.

The promotion of the crystal structure C-terminal conformation by lipid interactions may also reconcile some apparently conflicting results concerning the Tb-MscL C-terminal region. Previous MD simulations of the C-terminal region predicted that the region would only maintain its helical structure at low pH (or with neutralizing mutations) (Elmore and Dougherty, 2001). Similarly, individual peptides with the sequence of the Tb-MscL C-terminal region only showed significant helicity at a low pH. However, these peptides showed significant α-helicity at physiological pH when attached to a TASP system (Kochendoerfer et al., 2002). The TASP system started at residue 102 of the C-terminal region, which means that the TASP would have imposed structural constraints in the region involved with POPE-protein interactions. Conversely, the previous MD simulations of the C-terminal region alone included no lipid and only had moderate restraints on residues 94 and 95, more N-terminal than either the TASP attachment to the peptide or some residues that appear to mediate important lipid interactions in our present simulations. Thus, all these studies highlight that the structure and dynamics of the C-terminal region depend on the interplay of protein-protein and protein-lipid interactions in the region directly following TM2.

Extracellular Loop Region

In addition to the marked decrease in hydrogen bonding described above, the protein-lipid energy profile for the extracellular loop region shows decreased interactions after the change from POPE to POPC (Fig. 5.7A). Changes are particularly evident for two residues at the water/lipid interface, R45 and D68. This drop in protein-lipid interactions does not appear to be compensated by increased protein intersubunit interactions in POPC (Fig. 5.7B). However, despite these energetic changes resulting from the lipid headgroup alteration, no clear structural correlates were evident in the loop region in either measurements of RMS deviation and fluctuation or by visual inspection (data not shown). Since previous work has proposed functional importance



Figure 5.7: Protein-lipid (A) and intersubunit (B) interaction energies per residue for the extracellular loop region in POPE (black) and POPC (gray) simulations.

for this region in Tb-MscL (Maurer et al., 2000) and Ec-MscL (Ajouz et al., 2000), further site-specific dissection may implicate a functional importance of interactions modulated by lipid changes in this region. As well, unlike in the C-terminal region these Tb-MscL lipid interacting residues are not well conserved among MscL orthologues outside of the Mycobacteria (Maurer et al., 2000). In fact, D68 is aligned, or closely aligned, with a positively charged K or H residue in most non-Mycobacterial channels, including Ec-MscL. These differences could be related to the known physiological differences between Tb-MscL and other MscL channels (Moe et al., 1998; Moe et al., 2000).

Simulations with Lipid Tail Shortening

Overall System Adjustment to Lipid Shortening

To simulate the bilayer thinning that accompanies a rise in tension, we progressively shortened the lipid tails by successive removals of terminal methyl groups of the lipid chains. The lipid bilayer adjusted quite rapidly to the removal of each methyl group. Bilayer thickness, measured by the distance between phosphorous atoms on opposite sides of the bilayer, decreased quickly after each shortening step and generally remained level after a few hundred ps (Fig. 5.8A). On the average, each step thinned the membrane by 0.75-1 Å, which agrees with experimental measurements on lipid vesicle bilayers with varying tail lengths (Lewis and Engelman, 1983). In addition, the protein structure was not grossly altered in the simulations with lipid shortening, as shown by the RMS deviations between the trajectories and the starting crystal structure (Fig. 5.8B). The RMS deviation for all C α is relatively high in these simulations, primarily due to large deviations in the extramembrane regions. The extracellular loop was fairly disordered in the crystal structure, and the C-terminal region does not exhibit the lipid stabilization noted above when PME is not used to compute long-range electrostatics, leading to disorder similar to simulations of this region without lipid present (Elmore and Dougherty, 2001). As well, the RMS fluctuations (data not shown) of C α atoms in equilibrated systems were essentially identical, showing no clear regions of increased or decreased structural stability in the systems with shortened lipid.

Figure 5.8: A) Distance between the phosphorous atoms of the two lipid bilayer leaflets for all lipid shortening trajectories. This distance was calculated as the difference between the average z-axis positions of all phosphorous atoms in the leaflets. B) RMS deviation from crystal structure for the trajectories ending with 16/18 lipid (black), and 10/10 lipid (gray). These values were calculated as in Figure 3; values are reported for the RMS deviation of all C α and just the C α of the TM1/TM2 regions. The RMS deviation values for the trajectories ending in 14/14 and 12/12 were essentially the same as those shown.



Evidence of Hydrophobic Matching in the Simulations

Hydrophobic matching between MscL and the surrounding lipid has been proposed as a possible driving force for channel gating (Hamill and Martinac, 2001; Sukharev et al., 2001b). Both the lipid properties and protein movements in the lipid shortening trajectories exhibit clear signs of such hydrophobic matching. First, during membrane thinning, lipids that directly border the protein thin less than lipids in the bulk of the membrane (Fig. 5.9). Thus, it appears that favorable interactions with the protein

"restrain" the nearby lipids from compressing as much as those that are not directly coupled to the protein. Interestingly, in the 16/18 trajectory the opposite effect is observed, in that the bordering lipids are actually thinner than those in the bulk. This implies that the channel actually matches better to a lipid somewhat thinner than 16/18. Thus, despite the tendency of a lipid with a given tail length to form a bilayer of a particular thickness, hydrophobic matching with the channel causes lipids close to the channel to adopt a different thickness.



Figure 5.9: Average transbilayer distances between phosphorous atoms of lipids bordering Tb-MscL and in the bulk for lipid shortening simulations. The diagram on top schematically shows the difference between bordering and bulk lipids, with the channel depicted as a black rectangle and the lipid phosphorous atoms shown in gray. The average values for the final 1 ns of each lipid shortening simulation are given below along with the difference between values for the bordering and bulk lipids.

Changes in protein conformation in the simulations are also driven by hydrophobic matching. The majority of the channel-lipid interface consists of the second transmembrane domain and early portions of the C-terminal region. These regions can be used to define a "hydrophobic surface" on the outer face of Tb-MscL, as seen in Fig. 5.10. Although the exact definition of this surface is somewhat arbitrary, the analyses presented are robust to changing the definition by a few residues. The length of this hydrophobic surface along the bilayer normal (in the *z* direction) decreases during the membrane thinning simulations. Fig. 5.10 shows this length averaged over all five subunits for trajectories with membranes of different thickness. Clearly, these surfaces attempt to compress to better match the shortened lipid.

Figure 5.10: Average values for channel hydrophobic surface length (HSL) in lipid shortening simulations. The structure on the left denotes residues included in the "hydrophobic surface" of Tb-MscL, encompassing all of the TM2 helix and a portion of the C-terminus. Hydrophobic residues (Leu, Val, Ile, Ala, Phe, Tyr, Trp) are shown in blue, charged residues (Arg, Lys, Asp, Glu) are shown in red. The residues chosen as the termini of the "hydrophobic surface," L69 and



Y94, are displayed in spacefilling. The diagram on the top right shows the definition of HSL for a single subunit, shown as a blue line, with the lipid phosphorous atoms denoted in yellow. Average HSL values for all lipid shortening simulations are given on the bottom right.

Thus, hydrophobic matching leads to a balance between bordering lipid being restrained from full thinning and the channel decreasing its hydrophobic length. One can calculate the extent to which these combined effects compensate for the overall thinning of the bulk membrane from 16/18 to X/X using the expression:

% compensation = $\frac{\text{border lipid effect + HSL effect}}{\text{total bulk lipid thinning}} =$ $\frac{[\text{border PP}(X|X) - \text{bulk PP}(X|X)] + [\text{HSL}(16/18) - \text{HSL}(X|X)]}{\text{bulk PP}(16/18) - \text{bulk PP}(X|X)}$ where PP(X/X) is the average interleaflet P-P distance for the lipid X/X and HSL(X/X) is the hydrophobic surface length in the lipid X/X. Using this expression, the hydrophobic matching effects are 40-50% of the overall bulk membrane thinning in the 14/14, 12/12, and 10/10 trajectories, with the percentage decreasing for the increasingly thinner bilayers. Thus, the system shows only a partial compensation for the thinning effects. Perhaps more extensive lipid or protein perturbations would occur on a longer simulation timescale. Also, since the percent compensation decreased with more extensive thinning, the lipid and channel may have become increasingly decoupled from one another under such extreme shortening.

A border lipid effect also was observed in the POPC simulation described above. In the bulk, POPC thins considerably compared to POPE, from 44.2 Å to 38.1 Å. This thinning is in agreement with experimental comparisons between PE and PC lipids with identical hydrocarbon tails (Petrache et al., 2000a; Rand and Parsegian, 1989). However, our magnitude of thinning is somewhat greater than the 1-4 Å observed experimentally. This may result from bulk lipids overcompensating from the lack of thinning in border lipids, which are highly restrained, maintaining a thickness of 43.2 Å. The protein HSL showed no change in POPC vs. POPE. These simulations used the PME electrostatic model, and interestingly, the thinning of the border lipids noted above for the 16/18 POPE system was not seen when PME electrostatics were used. Thus, the inverse effect seen for 16/18 border lipids may have been an artifact of the less sophisticated cutoff electrostatics. However, the POPC results confirm that hydrophobic matching upon thinning is insensitive to the electrostatic method employed.

Structural Rearrangements Upon Bilayer Thinning

The decreased average hydrophobic surface length discussed above is not a consequence of a smooth, concerted motion of the 5 subunits of MscL, but rather results from especially large movements in one or two of the subunits. We considered the possibility that these motions could provide insights into the early conformational changes of Tb-MscL resulting from membrane thinning upon the application of tension to the membrane. One feature of the simulations was that pore constrictions were observed in the 14/14 and 12/12 shortening simulations (Fig. 5.11A). These trajectories exhibited a narrowed pore in the region near the V21 plug residue, extending to more extracellular portions of the pore.



B

Figure 5.11: A) Pore radius profiles for lipid shortening simulations calculated with HOLE. Frames taken every 50 ps for the final 250 ps of a trajectory were averaged for the profiles presented. The *z*axis position of each profile was adjusted such that V21 was at 0 nm. B) An example of a kinked TM2 helix from the final frame of the 10/10 trajectory. Our MD simulations also showed changes in TM2 conformation, and these contribute to the decreased hydrophobic surface length of the channel. However, these movements were not consistent between simulations. In some cases, the hydrophobic shortening resulted from tilting and/or kinking motions of the helical TM2 region (Fig. 5.11B). However, in other cases, thinning resulted from a compression of the less structured C-terminal portion of the hydrophobic surface. The potential role of TM2 kinking has been investigated using both additional MD simulations and experimental mutagenesis, as described below. This motion was focused on initially since helical kinking has been proposed in the gating of other ion channels (Tieleman et al., 2001).

Additionally, it is interesting to note that the channel seems to adjust its average hydrophobic length with relatively minor conformational changes. Thus, t he trajectories imply that although hydrophobic matching clearly occurs between Tb-MscL and the lipid membrane, this effect alone may not be sufficient to cause the major conformational changes leading to channel gating. This view is consistent with recent experiments showing that although MscL is easier to gate in vesicles with shorter lipid tail lengths, the channels still require some additional tension or perturbation in membrane structure to actually open (Perozo et al., 2002b).

Comparisons to Experimentally Derived Intermediate Gating Models

The motions observed in our lipid shortening simulations clearly do not lead to an open state of MscL. However, it is interesting to consider them in light of gating intermediates developed by Sukharev, Guy, and co-workers (Sukharev et al., 2001a; Sukharev et al., 2001b) and Perozo, Martinac, and co-workers (Perozo et al., 2002a;

Perozo et al., 2002b). The Sukharev/Guy intermediate model is much further along the gating pathway than any state we observe, making comparisons to it impossible. However, the relatively early Perozo/Martinac intermediate model, which was based on EPR measurements of spin-labeled MscL incorporated in thin (14/14) membranes, seems ideal for comparison. In fact, one significant feature of their model was a pore constriction analogous to that described above for the 14/14 and 12/12 trajectories (Perozo et al., 2002a). As well, this constriction was not observed for EPR measurements in the shortest lipids, similar to its disappearance upon shortening to 10/10 lipid in our simulations (Perozo et al., 2002b).

While this agreement between experiment and simulation is gratifying, another feature of the Perozo/Martinac model, a consistent TM1 rotation and tilt, was not observed in the simulations. Instead, extracellular portions of TM1 regions moved inwards towards the pore. Additionally, the Perozo/Martinac intermediate structure in 14/14 lipid does not propose any TM2 movements of the sort observed here. These differences between simulation and experiment could be a result of the relatively short timescale of these MD simulations. We note, however, that the model building used to interpret the experimental EPR was fairly simplistic, involving rigid helices and imposed five-fold symmetry (Perozo et al., 2002a). The MD simulations reported here do not involve such constraints and could therefore include helical deformations, such as TM2 kinking. Regardless, the idea of a slightly constricted intermediate state is intriguing, and determining its physiological relevance, if any, warrants further effort.

Correlations Between TM2 Energetic Profiles and Mutagenic Data

The protein-lipid and intersubunit interaction energy profiles for the Tb-MscL TM2 region both show distinct peaks that follow a helical pattern (Fig. 5.12A). This pattern occurs because one face of the TM2 helix faces the lipid bilayer, while another face interacts with the TM1 region of an adjacent subunit. These profiles are qualitatively unaltered by changes in lipid headgroup or lipid tail shortening.

Figure 5.12: A) Protein-lipid (yellow) and intersubunit (green) interaction energies per residue for the TM2 region of the POPE trajectory. Analogous profiles for the POPC and lipid shortening trajectories were essentially the same, showing identical energy peaks for protein-lipid and intersubunit energies. B) The correlation between Ec-MscL random mutagenesis data (Maurer and Dougherty, 2003) and MD interaction energies. The sequence alignment for Tb-MscL and Ec-MscL in the TM2 region is given on the two top rows. Below this, residues that are peaks for protein-lipid (yellow) and intersubunit (green) interactions in MD simulations are denoted. On the bottom three rows, results from random mutagenesis are summarized, showing Ec-MscL mutations which yielded GOF (blue), LOF (red), and wild-type-like (black) phenotypes.



Recent random mutagenesis studies of Ec-MscL have characterized several mutations in the TM2 region (Maurer and Dougherty, 2003). Many such mutations were determined to be gain-of-function (GOF), i.e., gating more easily than wild type, or loss-of-function (LOF), i.e., gating with more difficulty than wild type. Since the sequence homology between Ec-MscL and Tb-MscL is fairly high in this region (Maurer et al., 2000), we have compared this mutagenic data with our computed energetic profiles. In particular, we were interested in seeing whether residues that interact with lipid or those that interact with other subunits would be more sensitive to mutation. The experimental and computational results are summarized in Fig. 5.12B. Strikingly, almost all mutations that lead to an altered channel phenotype occur at positions in Ec-MscL that align to lipid interacting residues in Tb-MscL; this distribution is statistically significant by Fisher's exact test (p=0.042). An analogous correlation does not exist for residues that mediate intersubunit interactions (p=1.0).

These results imply that lipid interactions are more important than intersubunit interactions in determining normal MscL function. Mutation of lipid interacting residues could feasibly alter channel function in at least two manners. First, these mutations could affect interactions between the channel and surrounding lipid that are necessary for the transduction of tension between the bilayer and protein. Since MscL is known to gate when reconstituted alone in lipid vesicles (Häse et al., 1995), such protein-lipid interactions must be essential to transmitting the gating tension to the channel. However, the LOF phenotype observed for many mutations at lipid interacting residues could also result from improper assembly of the channel in the membrane, since a non-functional channel would appear as LOF in the assay used for characterization (Maurer and Dougherty, 2001). This possibility seems most likely for mutations that would place charged or polar residues directly next to the hydrophobic membrane if MscL assembled properly, for example Y75D. The importance of lipid interactions for proper channel assembly has been noted for other ion channels, such as KcsA (van Dalen et al., 2002).

Conversely, this analysis implies that residues which mediate MscL intersubunit interactions can be altered more readily without greatly affecting channel function. This may result from a redundancy in these interactions. There are several pairs of TM1-TM2 interactions that likely help transduce bilayer forces between the TM domains to gate the channel (Sukharev et al., 2001b), so other interactions may be able to compensate for the loss from a single mutation.

Investigation of the Role of TM2 Kinking in MscL Gating

Molecular Dynamics Simulations of F80P

The potential physiological relevance of the TM2 kinking behavior noted in simulations with gradually shortened lipid was probed further with a combination of MD simulations and experimental mutagenesis. First, we performed simulations of Tb-MscL with an F80P mutation in all five subunits (Fig. 5.13) to induce a kink in the middle of TM2 where a kink was observed in lipid shortening simulations. The F80P simulation showed an overall RMS deviation from crystal structure similar to that of other MscL simulations (data not shown), showing that the mutation did not appear to lead to an unreasonable trajectory. However, the channel did show significantly greater TM2 kinking in the F80P mutant trajectory (Fig. 5.14). While at almost all times after equilibration the wild-type structure would only have none or 1 kinked TM2 helix (as

evaluated using HELANAL (Bansal et al., 2000)), four or five of the helices typically were kinked after equilibration in the F80P trajectory (Fig. 5.14A). The proline-induced kink also leads to a notable "hinging" motion of the TM2 helices (Fig. 5.14B). Additionally, although the RMS *deviation* from crystal structure is similar for the TM helices in the wild-type and F80P trajectories, the RMS *fluctuations* of TM residues are higher in F80P (Fig. 5.15). Often an increase in RMS fluctuation, which is analogous to the temperature factor of a residue in crystal structures, is associated with increased structural instability in a region. Thus, this result hinted that a kink in TM2 might lead to structural destabilization of the closed state of MscL, thus promoting channel gating.



Figure 5.13: Single subunit from the Tb-MscL crystal structure with the F80 residue highlighted in red spacefilling.

However, the F80P simulation does not show characteristics associated with the putative "intermediate" structure from lipid shortening simulations. First, although the helices are generally kinked in the F80P simulation, they do *not* show a shorter

hydrophobic surface length (HSL) than the helices in the wild-type simulation (data not shown). Since TM2 kinking does not inherently cause HSL shortening, the shortening observed above as the channel adjusted to thinned bilayers was most likely not caused by TM2 kinking. Instead, the shortening of the HSL probably resulted from other channel conformational changes in the simulation. Second, the distinct pore narrowing observed both in lipid shortening simulations and the EPR studies of Perozo, Martinac and co-workers (Perozo et al., 2002a) is not observed in simulations of F80P (data not shown).

Figure 5.14: A) Histogram showing the number of saved frames during the last 1 ns with a given number of kinked TM2 helices for the wild-type and F80P simulations.
Helical kinking was measured with HELANAL. B) Picture of TM2 helices from frames taken every 100 ps over the last 1 ns of the F80P simulation. The Cα of residues 69-80 from the different frames were superimposed in the picture.



Together, these results imply that TM2 kinking is not likely to be a central feature of the MscL "intermediate" state proposed from experiments and computations with thinned lipid bilayers. In particular, inducing a kink in TM2 does not appear to drive the structure to such as intermediat. However, the RMS fluctuation results (Fig. 5.15) imply that mutations inducing such a kink might lead to interesting channel phenotypes. Thus, a series of experiments was performed to characterize how such kink-inducing proline mutations in TM2 would alter channel function.

Figure 5.15: RMS fluctuation of C α atoms per residue for TM1 (A) and TM2 (B) residues averaged over the last 1 ns of the wild-type (blue) and F80P (red) trajectories.



Experimental Characterization of Mutants with a TM2 Kink

To experimentally test the effect of transmembrane kinking, the *E. coli* (Ec) homologue of MscL was used since the very high gating tension of Tb-MscL makes its experimental characterization quite difficult. However, the generally high sequence homology between the TM domains of the two homologues (Maurer et al., 2000) makes comparisons between mutations in the TM2 region reasonable, such as the comparison of Tb-MscL computational results and Ec-MscL experimental results in the previous section. Thus, a series of Ec-MscL channels with a single proline mutation were made (D84P, F85P, L86P, I87P, V88P); Ec-MscL L86P is well-aligned with Tb-MscL F80P.

These residues encompass a full turn in the middle of TM2. All these mutations were made using the QuikChange Method (Stratagene) on a pB10b construct containing the Ec-MscL open reading frame. The mutants were functionally characterized using the Live/Dead BacLight method developed by Maurer and Dougherty and described in detail in (Maurer and Dougherty, 2001). In brief, bacterial cultures expressing mutant channels were subjected to a series of different osmotic downshock conditions, and the bacterial viability after downshock was measured using a fluorescent dye system. Since the MscL channel is thought to rescue bacteria from osmotic downshock, changes to the normal downshock response in bacteria expressing MscL mutants is attributed to changes in channel function. This method allows mutations to be characterized as having GOF, LOF, or wild-type-like phenotype.

Overall, all TM2 proline mutations led to a LOF channel (Table 5.1). These results further support the implication from the MD simulations that inducing TM2 kinking would not force the channel towards an intermediate state. One would expect that promoting the intermediate state of the channel would also make it easier for the channel to enter its open state, leading to a GOF phenotype. However, these results may at first seem contradictory to the RMS fluctuation results (Fig. 5.15) that showed a destabilization of the closed state resulting from TM2 kinking. However, a destabilization of the closed state would not necessarily imply that the channel would be pushed into the open state. Instead, it could be pushed into an unproductive non-native closed conformation. Looking at protein-lipid and intersubunit protein-protein interactions in the F80P simulation gives further insight into these possibilities.

Mutant	Phenotype
D84P	LOF
F85P	LOF
L86P	LOF
L86A	wt-like
I87P	LOF
V88P	LOF

Table 5.1: Experimentally characterized phenotypes of Ec-MscL TM2 mutants.

Intersubunit and Protein-Lipid Interactions of MscL TM2 Proline Mutants

Changes in the intersubunit interactions of TM2 residues in the F80P trajectory are consistent with the channel being pushed into a non-functional conformation by proline mutations. In the simulation, kinking at P80 brings the extracellular and intracellular ends of TM2 closer to TM1 (Fig. 5.16A). This closer proximity leads to a marked increase in intersubunit interactions of those TM2 residues (Fig. 16B). These increased interactions could effectively "glue" the ends of the two TM regions together, which would prevent the "sliding" motions of the TM helices proposed in the prevailing gating models of MscL (Perozo et al., 2002a; Sukharev et al., 2001a; Sukharev et al., 2001b). Thus, TM2 kinking induced by proline mutations may lock the channel into an occluded conformation, albeit one that is somewhat different than the native closed state.

A reduction in protein-lipid interactions could be another possible explanation for the LOF phenotype of TM2 proline mutations. The protein-lipid interaction profile of TM2 residues is very similar in the wild-type and F80P simulations with one clear exception—the mutation decreases the interaction of residue 80 with lipid (Fig. 5.17). Since analyses discussed above implied that altering MscL protein-lipid interactions often leads to altered channel function, the Ec-MscL L86P mutation could have caused a LOF phenotype by this mechanism instead of by TM kinking. To control for this possibility, a L86A Tb-MscL mutation that would also dramatically decrease protein-lipid interactions was made and characterized by the Live/Dead BacLight method. Since L86A showed a wild-type-like phenotype (Table 5.1), it appears that the LOF phenotype observed for L86P was more likely due to TM kinking and not to reduced protein-lipid interactions.

Figure 5.16: A) Representative interaction between a kinked TM2 helix (yellow) with TM1 in the adjacent subunit (green). The P80 residue is shown in red. B) Intersubunit interactions energies per residue for TM2 in the wild-type and F80P simulations.





Summary

Considering multiple lipid environments in MD simulations of MscL has led to insights into protein-lipid interactions that help interpret previous experiments and propose future work. Although not currently standard practice, similar consideration of multiple types of membrane may also yield interesting results for other membrane protein simulations. Certainly, the methods employed here could undergo further optimization, such as considering the effect of employing PME electrostatics in shortening simulations or extending simulations further. However, the systems were stable under our current protocols and adjusted quite quickly to the changes in lipid molecules.

In simulations comparing Tb-MscL in POPE and POPC lipids, POPE clearly seems to promote a C-terminal structure closer to the crystal structure conformation than that seen in POPC. Such differences in conformation could be one manner by which lipid composition affects Tb-MscL physiology. As well, lipid interactions and hydrogen bonding profiles were different for POPE and POPC in the extracellular loop region, although these differences did not correlate with clear structural changes in the simulations. Further experiments focusing on this region of the channel may clarify the importance of any such changes. Theoretical consideration of MscL in other types of lipid molecules using MD or other methods may lead to additional insights.

Trajectories in which lipid was gradually shortened showed that MscL does exhibit notable hydrophobic matching with lipid. Although these simulations did not lead to an open state of MscL—which would be shocking in the timescales considered-they did yield some hints of how the channel may adjust to membrane thinning. Such motions included constriction of the pore, which has also been observed experimentally (Perozo et al., 2002a), and kinking of TM helices. However, additional simulations and experimental mutagenesis showed that TM2 kinking is not likely an important feature of MscL intermediates. In fact, TM2 kinking led to a channel that was either non-functional or harder to gate than wild-type; increased interactions between TM1 and TM2 caused by kinking may have led to this LOF phenotype in TM2 proline mutants. Together, this data supports the use of rigid TM helices in the current gating models of MscL. It is also interesting to note that hydrophobic matching alone may only cause relatively small changes in channel structure, which would agree with data showing shorter lipids promote channel gating but are insufficient to induce channel gating by themselves (Perozo et al., 2002b).

Protein-lipid interactions in the TM2 region of MscL also showed intriguing correlations with experimental mutagenesis data (Maurer and Dougherty, 2003). In particular, residues that were seen in MD simulations to mediate protein-lipid interactions were mutagenically more sensitive than residues that mediated intersubunit interactions within the channel. This implies that protein-lipid interactions may be more important than protein-protein interactions for proper MscL assembly and function. It will be interesting to consider if this is a general theme for mechanosensitive membrane proteins.

Overall, this work has begun to give some molecular insight into how lipid composition affects MscL structure and function. The importance of lipid interactions in MscL gating has been discussed in efforts to develop gating models of MscL, but it has been difficult to address such interactions directly in these models (Sukharev et al., 2001b). Hopefully, combinations of experiments and theoretical methods, such as those employed here, will be fruitful in developing an increasingly complete picture of the interplay between protein and membrane in mechanosensitive channel gating, and membrane protein function in general.

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