CHAPTER 6

Lipid Effects on Mechanosensitive Channels

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I. OVERVIEW

The structure and function of a membrane protein depend on the properties of the lipid molecules, the annular lipid molecules, that surround it in a membrane. Fatty acyl chain length is important because lipid chain length determines the hydrophobic thickness of the lipid bilayer, and the efficiency of hydrophobic matching between a membrane protein and the surrounding lipid bilayer is high. Lipid headgroup structure can also be important because the lipid annulus can be heterogeneous with, for example, hot-spots where anionic lipids can bind with high affinity. An important question is whether

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the activity of a membrane protein depends only on the properties of the annular lipid molecules or is also dependent on the properties of the bulk lipid molecules in the membrane. Bulk properties that have been considered to be important include fluidity, curvature stress, elastic strain, and transmembrane pressure profiles. This chapter considers to what extent these general features of lipid–protein interactions help us to understand the properties of the bacterial mechanosensitive channel of large conductance (MscL).

II. INTRINSIC MEMBRANE PROTEINS

All membrane proteins operate in the environment of a lipid bilayer and so are likely to be affected by changes in the chemical composition or physical properties of the lipid bilayer. The bacterial mechanosensitive channels are unusual membrane proteins in that their dependence on the lipid bilayer is the key to their function: It is an increase in the tension in the lipid bilayer that leads to channel opening (Moe and Blount, 2005). However, insights into how bacterial mechanosensitive channels might function are likely to come from studies of other classes of membrane protein because lipid–protein interactions are important for all membrane proteins. Some membrane proteins require specific lipids for function, these lipids binding to specific sites on the membrane proteins (Lee, 2003, 2004, 2005). The effects of such lipids clearly need to be understood in terms of the specific interactions between the bound lipid molecules and the membrane protein. However, the majority of the lipids around a membrane protein will not interact with the protein in this way, playing a role more like a conventional solvent molecule.

The first question that needs to be answered is whether the function of a membrane protein changes when the structures of the "solvent" lipid molecules surrounding it in a membrane are changed. If protein function is found to be sensitive to the nature of the surrounding lipids, then the question arises as to whether this dependence is best explained at the molecular level, in terms of specific molecular interactions between the lipids and proteins, or at the macroscopic level, in terms of bulk properties of the lipid bilayer.

III. EFFECTS OF LIPID STRUCTURE ON MEMBRANE PROTEIN FUNCTION

That membrane protein function is sensitive to lipid structure is most readily demonstrated for membrane-bound enzymes, since the techniques of presteady-state and steady-state kinetics allow detailed insights into mechanism (Michelangeli *et al.*, 1991). Figure 1 shows the effects of lipid

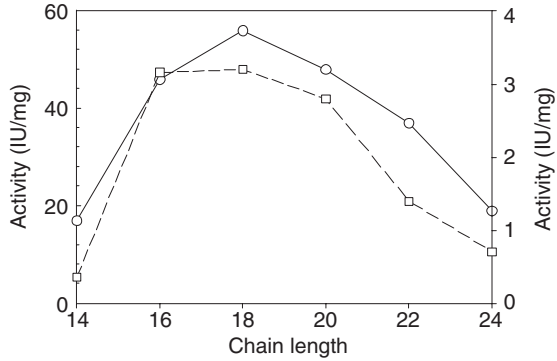

FIGURE 1 The effect of fatty acyl chain length on enzyme activity in bilayers of phosphatidylcholine in the liquid crystalline phase. Ca^{2+}-ATPase (□; right-hand axis) or diacylglycerol kinase (○; left-hand axis) were reconstituted into phosphatidylcholines containing monounsaturated fatty acyl chains of the given chain lengths. ATPase activities were determined at 25°C. For diacylglycerol kinase, the substrate was DHG present at 20 mol% in the bilayer. Data from Pilot *et al.* (2001a) and Lee (2003).

fatty acyl chain length on the activities of two membrane-bound enzymes, the Ca^{2+}-ATPase from skeletal muscle sarcoplasmic reticulum (Froud *et al.*, 1986a,b; Starling *et al.*, 1993) and the bacterial enzyme diacylglycerol kinase (DAGK) that uses ATP to phosphorylate a diacylglycerol to give the corresponding phosphatidic acid (Pilot *et al.*, 2001a). In both cases, highest activity is seen in bilayers of phosphatidylcholines in the liquid crystalline phase when the fatty acyl chain length is C18, with lower activities in bilayers of lipids with shorter or longer fatty acyl chains. However, the reasons why short- and long-chain lipids give low activities for the Ca^{2+}-ATPase are different from the reasons why they give low activities for DAGK, and the reasons for the low activities in short-chain lipids are different from the reasons for the low activities in long-chain lipids. For example, the low rate of ATP hydrolysis observed for DAGK in di(C14:1)PC (Fig. 1) follows from a very high K_m value for dihexanoylglycerol (DHG) in di(C14:1)PC, the value for v_{max} being the same as in di(C18:1)PC (Pilot *et al.*, 2001a) (Fig. 2). In contrast, the low activity measured for DAGK in di(C24:1)PC follows from a low value for v_{max}, the value for K_m being comparable to that in di(C18:1)PC (Pilot *et al.*, 2001a) (Fig. 2). For the Ca^{2+}-ATPase, the low rate of ATP hydrolysis in di(C14:1)PC follows in large part from a slow rate of phosphorylation of the Ca^{2+}-ATPase by ATP (Starling *et al.*, 1995a) (Fig. 3). In di(C24:1)PC, the rate of phosphorylation is the same as that in di(C18:1)PC (Fig. 3) and the low rate of ATP hydrolysis by the Ca^{2+}-ATPase in di(C24:1)PC follows from a slow rate of dephosphorylation of

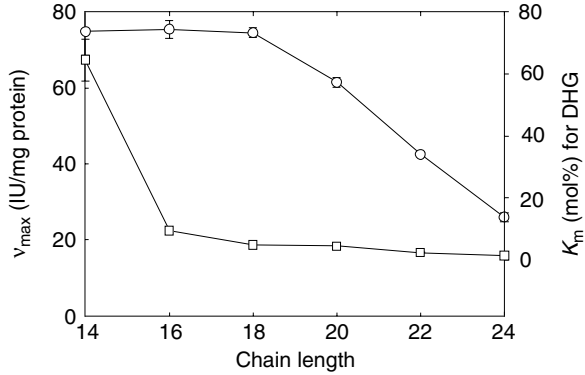

FIGURE 2 Effects of phosphatidylcholine chain lengths on K_m and v_{max} values for DHG. v_{max} (○) and K_m (□) values for DHG (expressed as mol% DHG in the membrane) at a fixed ATP concentration of 5 mM are plotted vs chain length. Data from Pilot *et al.* (2001a).

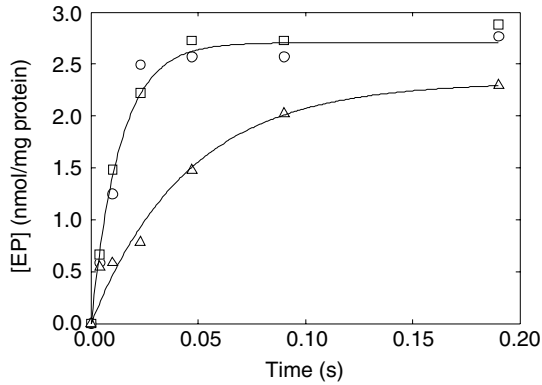

FIGURE 3 Rate of phosphorylation of Ca^{2+}-ATPase as a function of fatty acyl chain length. The Ca^{2+}-ATPase was reconstituted in di(C18:1)PC (○), di(C24:1)PC (□), or di(C14:1)PC (Δ) in the presence of Ca^{2+} and mixed with ATP to give a final concentration of 50 μM, and the level of phosphorylation was determined at the given times. The lines show fits to single exponential rate processes. Data from Starling *et al.* (1995a).

the phosphorylated intermediate (Starling *et al.*, 1995a,b). Effects of lipid chain length on the properties of the Ca^{2+}-ATPase are particularly complex, most, if not all, of the steps in the reaction sequence being affected, the most surprising change being a change in the stoichiometry of Ca^{2+} binding to the Ca^{2+}-ATPase, from 2:1 in the native membrane or in di(C18:1)PC, to 1:1 in short- or long-chain lipid (Michelangeli *et al.*, 1990; Starling *et al.*, 1993).

Lipid headgroup structure also has important effects on activity. The activity of the Ca^{2+}-ATPase is lower in a phosphatidylethanolamine than in the corresponding phosphatidylcholine, largely due to a decreased rate of dephosphorylation of the phosphorylated intermediate (Starling *et al.*, 1996a). The Ca^{2+}-ATPase also has low activity in bilayers of anionic lipids (Dalton *et al.*, 1998) and the activity of DAGK is also low in bilayers of phosphatidylethanolamines and anionic lipids (Pilot *et al.*, 2001b). Studies of the Ca^{2+}-ATPase in mixtures of short-chain phosphatidylcholines and normal-chain anionic lipids suggest that the effects of lipid headgroup and lipid fatty acyl chain on protein function can be considered to be separate (Dalton *et al.*, 1998). It has been suggested that changes in lipid structure could affect protein function by changing the aggregation state of the protein, but this has been shown not to be the case for the Ca^{2+}-ATPase (Starling *et al.*, 1995b).

A number of conclusions can be drawn from these results. The first is that there is unlikely to be any one, unique, explanation for the effects of lipid structure on membrane protein function. The second is that changing lipid structure results in changes in protein structure, the changes in protein structure underlying the observed changes in function. Thus, membrane proteins are not rigid, but have a degree of plasticity, allowing them to deform to help provide optimum matching to the surrounding lipid bilayer.

IV. HOW TO EXPLAIN EFFECTS OF LIPID STRUCTURE ON MEMBRANE PROTEIN FUNCTION

A. The Lipid Annulus

To what extent can the structures and functions of membrane proteins be explained using the language and principles developed for water-soluble proteins? Explanations for the effects of solvent water on the structure and function of a water-soluble protein are based largely on the hydrophobic effect and on hydrogen bonding between water molecules and polar residues on the solvent-exposed surface of the protein; explanations rarely involve bulk properties of the water such as viscosity or the "pressure" exerted on the protein molecule as a result of collisions of the water molecules with the protein surface. Can the effects of the lipid molecules that cover the transmembrane surface of a membrane protein also be understood in the same way, as simple solvent effects? Certainly, crystal structures of membrane proteins containing resolved lipid molecules show the importance of hydrogen bonding and charge interactions between polar or charged residues on the protein and the lipid headgroup and backbone region (Lee, 2003).

Changing lipid headgroup structure, leading to changes in the interaction between the lipid headgroup and the protein, could well lead to changes in protein structure and thus to changes in protein function.

The solvent lipid molecules covering the hydrophobic surface of a membrane protein are generally referred to as boundary or annular lipids, as they form an annular shell around the protein (Lee, 2003, 2004, 2005). The importance of this annular shell has been shown in experiments with the Ca^{2+}-ATPase; the number of lipid molecules required to form an annular shell around the Ca^{2+}-ATPase is about 30 (East *et al.*, 1985), and the activity of the Ca^{2+}-ATPase remains constant as the number of lipid molecules per Ca^{2+}-ATPase molecule is reduced from 90, corresponding to the lipid:protein molar ratio in the native membrane, to about 30, below which activity declines (Warren *et al.*, 1974). The fact that activity is maintained in a membrane with essentially no bulk lipid shows that the presence of bulk lipid is not essential for activity and thus that the properties of the bulk lipid are less important determinants of activity than the properties of the annular lipid: In other words, the annular lipid molecules largely buffer a membrane protein from the effects of bulk lipid.

B. The Fluidity of a Lipid Bilayer and Its Consequences

The lipid bilayer component of a biological membrane is generally in a fluid state, commonly referred to as the liquid crystalline state. This is marked by considerable motional freedom of the lipid molecules. Figure 4 illustrates the types of motion that we might expect in a lipid bilayer in the

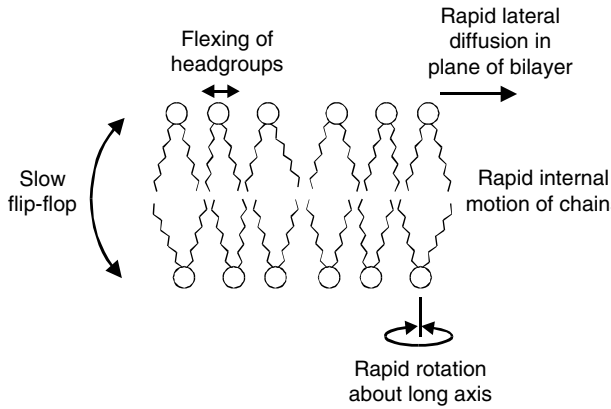

FIGURE 4 Classes of motion in a phospholipid bilayer.

liquid crystalline phase. The most important of the intramolecular motions is rotation about C—C bonds in the fatty acyl chains and in the headgroup region; it is this that makes the lipids "liquid-like." Motions of the whole lipid molecule include a fast lateral diffusion in the plane of the membrane and a fast axial rotation of the lipid about its long axis. Flip-flop motion in which a lipid moves from one side of the membrane to the other is, however, slow, since it involves moving the polar headgroup of the lipid through the hydrocarbon core of the membrane; it is the slowness of this motion that allows an asymmetric distribution of lipids between the two halves of a biological membrane.

In some ways the interior of a lipid bilayer is like a simple hydrocarbon. For example, an analysis of NMR spin-lattice relaxation times has suggested that *trans-gauche* isomerization rates (10^9–10^{10} s^{-1}) in fatty acyl chains in a lipid bilayer are very similar to those in a free chain and that the effective viscosity for the bilayer is ca. 0.01 P (Poastor *et al.*, 1988). This can be compared with the viscosity of neat hexadecane at 50°C, which is 0.019 P (Small, 1986). Similarly, molecular packing of the chains in the liquid crystalline bilayer is equivalent to that of a liquid alkane, with average methylene and methyl group volumes in the bilayer interior of 28 and 54 $Å^3$, respectively (Petrache *et al.*, 1997), comparable to the methylene and methyl group volumes in a liquid alkane of 27 and 57 $Å^3$, respectively (Nagle and Wiener, 1988). Nevertheless, there are important differences between a lipid bilayer and a normal liquid. The chemical nature of the lipid molecule, with fatty acyl chains "anchored" at the top of the chain to a relatively immobile backbone, most commonly a glycerol group, results in a gradient of motion along the lipid fatty acyl chains. A proper description of this chain motion requires a clear distinction to be made between the rates and the amplitudes of motion. The term "fluidity" and its inverse, viscosity, refers strictly, only to the rate of motion but in the biological literature the word fluidity is used very informally to include both factors. This is largely a reflection of the fact that most methods used to measure rates of motion (NMR, ESR, fluorescence depolarization) are, in fact, sensitive to both rates and extents of motion, and separating the two can be difficult. The extent or range of motion is described in terms of an *order parameter* that describes the time-averaged disposition in space of each group of atoms in the fatty acyl chain. The rate of motion can be described in terms of a *correlation time*, a measure of the rate of movement of a group of atoms between its various possible positions in space.

The idea of an order parameter can be introduced by considering the lipid molecules in a bilayer to be long, thin cylinders (Fig. 5). In a bilayer, the long axes of the cylinders will tend to align about a direction normal to the surface of the bilayer. At finite temperatures, the thermal motions of the

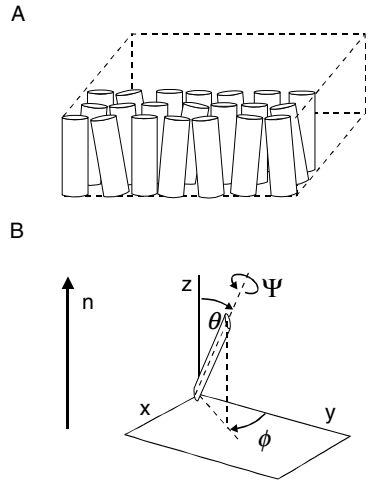

FIGURE 5 Orientation of lipid molecules (represented as cylinders) in one leaflet of a bilayer (A) and the angles required to describe the orientation of a molecule in the bilayer (B).

molecules will prevent the alignment from being perfect; the cylinders will occupy a range of angles about the direction of the bilayer normal. In Fig. 5, the bilayer normal is aligned along the z-axis of a fixed rectangular coordinate system. The orientation of the cylinder can then be described by the three angles shown. The cylindrical symmetry of the molecules means that there can be no order in the system in the angle ψ (describing rotation of the cylinder about its long axis) or in the angle φ (describing rotation of the cylinder about the z direction): If any particular angle ψ or φ had been preferred in some way, then the shape of the molecule could not have been cylindrical. However, a degree of order can exist for the angle θ. The most likely value of θ for the bilayer structure will be 0°, with the long axis of the cylinder parallel to the bilayer normal. However, since order is not perfect, a range of values of θ will be observed, centered around this most probable value of 0°. In contrast, in a normal isotropic liquid, where no direction in space is preferred, all values of the angle θ will be equally likely.

The angle θ itself, although a measure of order, is not a very convenient one. Rather, it is normal to introduce an order parameter, a function of $\cos^2\theta$. Actually, it is not the instantaneous value of $\cos^2\theta$ for one given molecule that is important, but rather the averaged value of $\cos^2\theta$ over the timescale of the measurement for all the molecules in the sample; this average is written as $<\cos^2\theta>$. Finally, it is traditional to write an order parameter in such a way that it has the value of 1 for a perfectly ordered

sample and a value of 0 for a completely disordered isotropic liquid. For a fully ordered system, $\theta = 0^0$ for all molecules and so the value of $<\cos^2\theta>$ will be 1, but for a totally disordered system when all values for θ are equally likely, the value of $<\cos^2\theta>$ is 1/3. Thus, the order parameter S that is used is:

$$S = \frac{3 < \cos^2\theta > -1}{2} \tag{1}$$

This has the required properties: $S = 1$ for a completely ordered system and $S = 0$ for the isotropic phase. The formalism described above is that used to describe the order of a C—H bond in a fatty acyl chain of a lipid molecule in terms of the average angle between the C—H bond and the bilayer normal. Anchoring the fatty acyl chains of a phospholipid molecule at one end to the lipid backbone results in a gradient of motion along the chain, the extent or range of motion increasing from the backbone to the terminal methyl end of the chain. Each CH_2 group in the chain will, therefore, have its own characteristic range of motion and thus its own order parameter. The most powerful technique for measuring these order parameters is ^{2}H NMR, studying the motion of C—D groups introduced at specific positions in the chains (Seelig and Seelig, 1980; Bloom *et al.*, 1991).

The order parameter of the C—D bond, S_{CD}, is defined as

$$S_{CD} = \frac{3 < \cos^2\Phi_j > -1}{2}, \tag{2}$$

where Φ_j is the angle between the bilayer normal and the C—H vectors at carbon atom j. S_{CD} describes the extent of the time-averaged excursions experienced by the C—H group.

For an all-*trans* configuration of a saturated fatty acyl chain rotating about the bilayer normal, the value of S_{CD} will be -0.5. At higher temperatures, *trans-gauche* isomerizations about the C—C bond leads to a mixture of *trans* and *gauche* configurations along the chain, which will reduce the absolute value (magnitude) of S_{CD}. The interpretation of the experimental order parameter S_{CD} in molecular terms may not always be as simple as suggested above since measured value of S_{CD} can also depend on the geometry of the deuterated molecule. This can be important for chains containing C=C double bonds, since the orientation of a C—D bond for a C atom in a double bond will necessarily be different to that in a normal CH_2 group.

Order parameter profiles for all phospholipid bilayers in the liquid crystalline phase are very similar. That for the palmitoyl chain in 1-palmitoyl-2-oleoylphosphatidylcholine [(C16:0, C18:1)PC] is shown in Fig. 6 (Seelig and

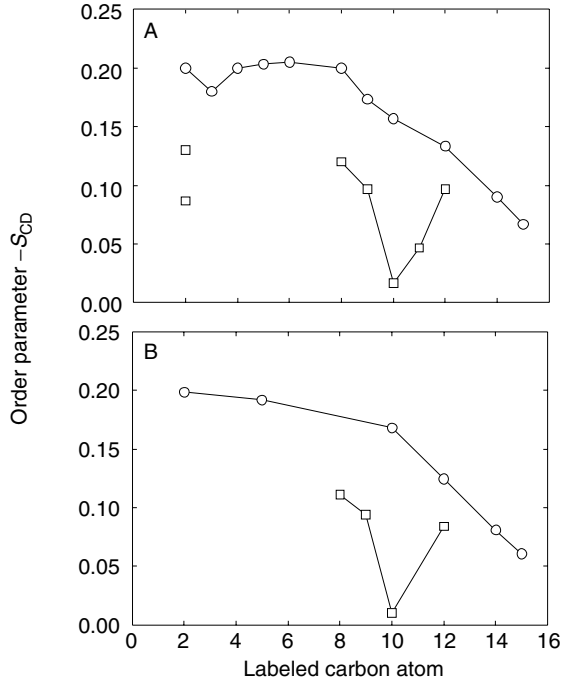

FIGURE 6 The experimental order parameters ($-S_{CD}$) for the palmitoyl (○) and oleoyl (□) chains of (A) (C16:0, C18:1)PC; (B) lipids of *E. coli* labeled in the palmitoyl (○) and oleoyl (□) chains. Data from Seelig and Seelig (1980).

Seelig, 1980). The measured order parameters are observed to lie between the values expected for an all-*trans* chain rotating about its long axis ($S_{CD} = -0.5$) and for complete orientational disorder, as found in an isotropic liquid ($S_{CD} = 0$). Thus, some order persists in the fatty acyl chain region despite the liquid-like state of the chains. The degree of order varies along the chain, an initial plateau region of constant order being followed by a region of rapidly decreasing order toward the center of the bilayer. The plateau region has its origin in intermolecular restrictions on chain motion. Excluded volume effects are very important in the upper part of the chain since rotation about a single C—C bond results in a large bend in the chain. Lower down the chain, lateral displacements resulting from rotations about single C—C bonds are very much smaller, and, therefore, steric restrictions on motion become less important.

The order parameter profile for the unsaturated chain in (C16:0, C18:1)PC appears to be very different to that for the saturated chain with the

experimental order parameters for carbon atoms 10 and 11 being low in the oleoyl chain (Fig. 6). This does not, however, indicate a high degree of motional disorder for these carbons, but rather follows from effects of the *cis* double bond on the orientation of C—D bonds with respect to the bilayer normal. Nevertheless, molecular dynamics simulations do show an increased motion for the C=C double bond and the methylene groups next to it, particularly for that on the terminal methyl side (Heller *et al.*, 1993; Huang *et al.*, 1994). Increased disorder in the region of the double bond is the result of shallow energy barriers for rotation about C—C bonds adjacent to a double bond (Li *et al.*, 1994).

The effects of polyunsaturation have also been studied in a series of phosphatidylcholines with a deuterated stearoyl chain at the *sn*-1 position and an unsaturated chain at the *sn*-2 position. Effects are rather small, the *sn*-1 chain becoming slightly more disordered as the unsaturation of the *sn*-2 chain is increased, but with the effect of unsaturation reaching a maximum at three double bonds (Holte *et al.*, 1995). There has been special interest in the properties of lipids containing polyunsaturated docosahexaenoic acid (DHA) chains because of its high concentration in retinal rod membranes. The DHA chain shows considerable flexibility because of the large number of *cis* double bonds that it contains (Feller *et al.*, 2002), and it has been suggested that the extreme flexibility for the DHA chain could be important for interaction with membrane proteins (Grossfield *et al.*, 2006). A molecular dynamics simulation of rhodopsin in a bilayer of 1-stearoyl-2-docosohexaenoyl-phosphatidylcholine showed that the DHA chains penetrate deeper into the protein interface than do the stearic acid chains (Grossfield *et al.*, 2006). It was suggested that the extreme flexibility of the DHA chain could allow it to adapt better to the rugged surface of the protein (Grossfield *et al.*, 2006).

Profiles of chain order are largely unaffected by lipid headgroup, although absolute values of order parameters can be affected. Thus, order parameters for phosphatidylethanolamines in the liquid crystalline phase are almost constant for the first part of the chain, but decrease rapidly toward the terminal methyl group, as for the phosphatidylcholines, but the order parameters are higher for phosphatidylethanolamines than for phosphatidylcholines, at all positions of the chain (Perly *et al.*, 1985; Lafleur *et al.*, 1990). The higher order parameters in phosphatidylethanolamines can be attributed to the smaller headgroup of the phosphatidylethanolamine and to strong intermolecular hydrogen bonding between the headgroups, both factors leading to a greater packing density throughout the bilayer. However, differences in packing density between phosphatidylcholines and phosphatidylethanolamines in the chain region must be quite small since the thicknesses of bilayers of di(C18:1)PC and di(C18:1)PE are equal (Fenske *et al.*, 1990).

An important observation is that order parameter profiles for intact biological membranes are the same as for those for simple lipid bilayers. The profiles for *Escherichia coli* membranes labeled in the palmitoyl and oleoyl chains are shown in Fig. 6; the values of the order parameters and their variation along the chain are the same as in bilayers of (C16:0, C18:1) PC (Seelig and Seelig, 1980). Thus, the presence of membrane proteins has no significant effect on the extent of motion of the average lipid fatty acyl chain in the membrane. A second important point also follows from these results that the lipids in the *E. coli* inner membrane must adopt a bilayer structure since the order parameter profile is that characteristic of a bilayer, despite the fact that the predominant lipid in the *E. coli* inner membrane is phosphatidylethanolamine, a lipid that, on its own, would prefer the hexagonal H_{II} phase. This is consistent with model system studies which show that even a small proportion of lipids favoring a bilayer structure will stabilize nonbilayer favoring lipids in a bilayer structure (Boni and Hui, 1983).

There is also considerable motion in the lipid headgroup region in the liquid crystalline phase. Average orientations of the headgroups in phosphatidylcholines and phosphatidylethanolamines are roughly parallel to the bilayer surface in the liquid crystalline phase (Buldt and Wohlgemuth, 1981), but molecular dynamics simulations for phosphatidylcholines show that the orientations of the P–N vectors in individual molecules can vary from an angle of zero with respect to the bilayer normal, so that the NMe_3^+ group is pointing out into the solvent, to values greater than 90°, so that the NMe_3^+ group is pointing into the hydrocarbon core of the bilayer (Heller *et al.*, 1993; Stouch *et al.*, 1994; Hyvonen *et al.*, 1997).

Proper function of a membrane protein generally seems to require that the lipid bilayer be in a liquid crystalline phase but there is, however, no compelling evidence to suggest that the exact fluidity in the fluid crystalline phase is important for function (East *et al.*, 1984; Lee, 1991). The importance of the liquid crystalline phase is that it gives the bilayer the important property of plasticity; the relatively weak interactions between neighboring lipid molecules means that the bilayer can distort around a foreign body, without any large-scale breakdown of bilayer structure. For example, Fig. 7 shows the results of a molecular dynamics simulation of the hemagglutinin fusion peptide bound to a bilayer of (C16:0, C18:1)PC (Lague *et al.*, 2005). Effects are largely limited to the immediate neighbors of the peptide; the presence of the peptide has no effect on the properties of the bulk of the lipids in the bilayer. On the peptide-containing side of the bilayer, the chains immediately adjacent to the peptide become more disordered (S_{CD} values are low) and thus shorter, as the chains curl to fill the space under the peptide. In contrast, chains immediately below the peptide in the other monolayer become more ordered and thus longer, and extend into the upper monolayer,

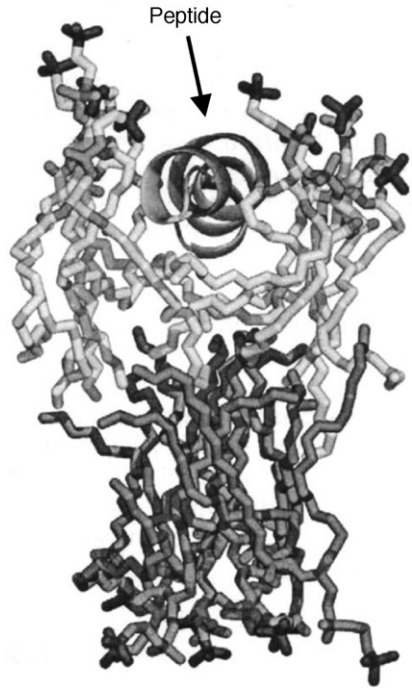

FIGURE 7 Packing of (C16:0, C18:1)PC around the hemagglutinin fusion peptide. Modified from Lague *et al.* (2005).

helping to fill the space under the peptide (Lague *et al.*, 2005). Similar results were obtained in a molecular dynamics simulation of a simple tripeptide binding to a bilayer surface, where again the bilayer was able to accommodate the peptide without significant change to the properties of the bulk phospholipids (Damodaran *et al.*, 1995). This high degree of plasticity means that the bilayer will provide little resistance to a change in shape for a membrane protein, allowing the protein to undergo any conformational changes required for function.

C. *The Importance of Hydrophobic Thickness*

An important way in which a lipid bilayer differs from a bulk solvent like water is in possessing a distinct thickness. Given that the cost of exposing hydrophobic regions of either a lipid bilayer or a protein to water is high, it can be expected that the hydrophobic thickness of a membrane protein will

match closely that of the surrounding lipid bilayer. This has been shown in dramatic fashion for the potassium channel KcsA where Trp residues at the ends of the transmembrane α-helices have been shown to maintain their positions close to the glycerol backbone region in bilayers of phosphatidylcholines over a chain length range C12-C24, representing a more than twofold change in bilayer hydrophobic thickness (Williamson *et al.*, 2002). The two most likely ways in which this hydrophobic matching could be achieved are illustrated in Fig. 8. The first involves compression or stretching of the lipid fatty acyl chains around the membrane protein to achieve matching. However, compressing or stretching the lipid bilayer requires work and, if the required work is sufficiently high, it may become more favorable energetically to distort the protein; if a membrane protein can exist in states with different hydrophobic thicknesses, then the equilibrium between these states will be shifted toward the state whose hydrophobic thickness best matches the hydrophobic thickness of the unperturbed surrounding lipid bilayer, so minimizing the requirement to distort the lipid bilayer. For example, gramicidin is a small peptide that dimerizes to form channels across the membrane; the hydrophobic length of the gramicidin dimer is relatively short and so thick lipid bilayers shift the monomer-dimer equilibrium toward monomer (Lundbaek and Andersen, 1999; Lundbaek *et al.*, 2004). For an intrinsic membrane protein, the most obvious distortion to achieve hydrophobic matching is tilting of the transmembrane α-helices (Fig. 8). Such tilting was

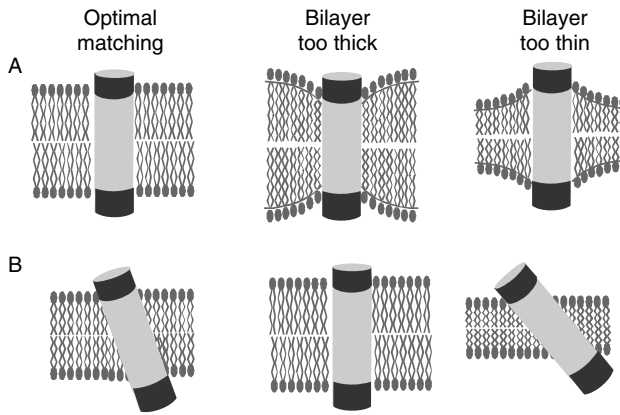

FIGURE 8 Possible responses to hydrophobic mismatch between a membrane protein and its surrounding lipid bilayer. In (A) matching to a too thick or a too thin bilayer results from compressing or stretching of the lipid fatty acyl chains, respectively. In (B) matching to a too thick or a too thin bilayer results from decreasing or increasing the tilt angle of a transmembrane α-helix, respectively.

suggested to explain the behavior of simple model transmembrane helices in lipid bilayers (Webb *et al.*, 1998), in agreement with molecular dynamics simulations (Kandasamy and Larson, 2006). Tilting of transmembrane α-helices to minimize hydrophobic mismatch has been detected experimentally for the pore region of the M2 proton channel (Duong-Ly *et al.*, 2005) and for the protein Vpu from HIV-1 (Park and Opella, 2005).

An important observation is that effects of bilayer thickness on membrane protein function are cooperative. This is shown in Fig. 9 for the Ca^{2+}-ATPase in mixtures of di(C18:1)PC and di(C14:1)PC; the changes in Ca^{2+}-binding stoichiometry and ATPase activity characteristic of short-chain lipid only occur when the bilayer contains more than 50 mol% of the short-chain lipid (Starling *et al.*, 1993). Since the binding affinity of the Ca^{2+}-ATPase for lipid varies little with chain length (East and Lee, 1982), the results illustrated in Fig. 9 show that the structural changes in the Ca^{2+}-ATPase leading to low activity only occur when more than about 15 of the 30 lipids in the annular shell of the Ca^{2+}-ATPase (East *et al.*, 1985) are short-chain lipids; the energy required to distort one lipid molecule might be less than that required to distort the Ca^{2+}-ATPase, but the energy required to distort 15 lipid molecules is presumably comparable to that required to distort the Ca^{2+}-ATPase.

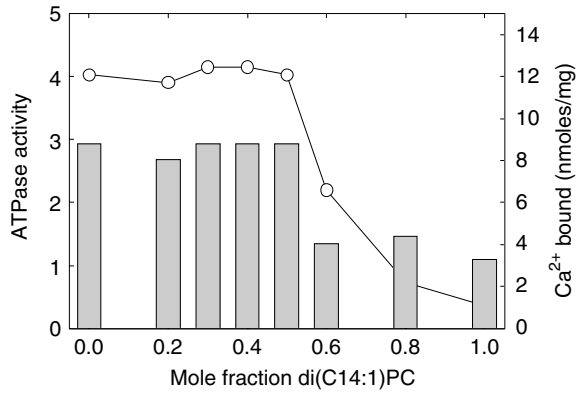

FIGURE 9 Effects of mixtures of di(C14:1)PC and di(C18:1)PC on Ca^{2+} binding and activity of the Ca^{2+}-ATPase. The Ca^{2+}-ATPase was reconstituted with mixtures of di(C14:1)PC and di(C18:1)PC containing the given mole fraction of di(C14:1)PC. The bars show the levels of Ca^{2+} binding (nmol Ca^{2+} bound/mg protein). Also shown are the ATPase activities measured at 25°C (○). Data from Starling *et al.* (1993).

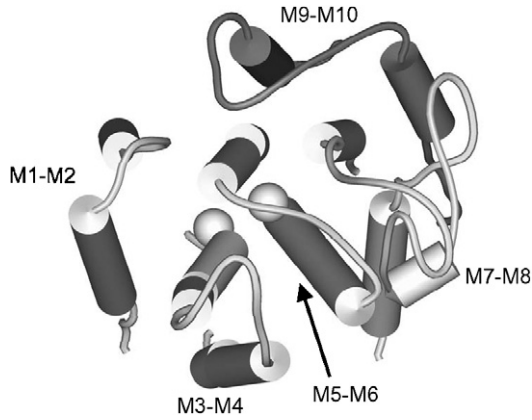

FIGURE 10 Loops between transmembrane helices on the luminal side of the Ca^{2+}-ATPase in its Ca^{2+}-bound form. The view is end-on, from the luminal side of the membrane. The locations of the loops between transmembrane helices M1-M2, M3-M4, M5-M6, M7-M8, and M9-M10 are marked. The two bound Ca^{2+} ions are shown in space fill format (PDB file 1EUL).

It is possible that the structure of the Ca^{2+}-ATPase makes distortion of the structure relatively easy (Lee, 2002). Loops connecting the transmembrane α-helices on the luminal side of the Ca^{2+}-ATPase are short, the loops making little contact with each other (Fig. 10), so that it will be largely the relative strengths of the helix–helix and helix–lipid interactions that keep the transmembrane helical bundle of the Ca^{2+}-ATPase intact. Changing the lipid composition around the Ca^{2+}-ATPase could, therefore, lead to changes in the packing of the transmembrane helical bundle, likely to result in changes in function since this is a critical region for the Ca^{2+}-ATPase, containing the two Ca^{2+}-binding sites on the Ca^{2+}-ATPase.

D. Curvature Stress

Lipids with small polar headgroups, such as the phosphatidylethanolamines, are said to have a "conical" shape in contrast to a lipid such as a phosphatidylcholine with a larger headgroup, which is said to have a "cylindrical" shape. Whereas lipids with a cylindrical shape will pack into a planar bilayer structure, a conically shaped lipid has a tendency to form curved, hexagonal H_{II} phases (Cullis and de Kruijff, 1979). In a biological membrane, the presence of both the intrinsic membrane proteins and bilayer-preferring lipids will, however, force lipids such as the phosphatidylethanolamines to adopt a bilayer structure (Lee, 2004) and the lipid will, therefore, be in a state

of curvature stress. However, although the concept of a "conical" shape for a phosphatidylethanolamine is helpful in considering the phase preferences of the isolated lipid, it would not be accurate to say that a phosphatidylethanolamine adopted a "conical" shape when in a lipid bilayer; if it did, the presence of a phosphatidylethanolamine in a bilayer of phosphatidylcholine would create a greater packing density toward the center of the bilayer and a smaller packing density near the glycerol backbone region and thus increase order parameters for phosphatidylcholine chains at the terminal methyl ends of the chains and decrease order parameters at the carboxyl end. Such effects are not seen, addition of a phosphatidylethanolamine to a bilayer of a phosphatidylcholine increasing order parameters at all positions in the chains of the phosphatidylcholine (Fenske *et al.*, 1990). Further, it has been shown that, in mixtures of (C16:0, C18:1)PE and (C16:0, C18:1)PC, the order parameters for the palmitoyl chains in (C16:0, C18:1)PE and (C16:0, C18:1)PC are the same (Lafleur *et al.*, 1990). This is another example of the important plasticity of the lipid bilayer.

It has been suggested that the curvature elastic energy stored in a membrane could shift the equilibrium between conformational states of an intrinsic membrane protein to that with the greatest hydrophobic thickness (Fig. 11) (Botelho *et al.*, 2002). The presence of a lipid such as phosphatidylethanolamine favoring negative curvature would favor the conformational state with largest hydrophobic thickness (Fig. 11). Extensive studies of the effects of hydrophobic additives on the function of a sodium channel were shown to be consistent with such a model (Lundbaek *et al.*, 2004). However, it is not yet clear how common will be large differences in hydrophobic thickness between the different conformational states of a membrane protein. For the Ca^{2+}-ATPase where crystal structures are available for a number of conformational states (Moller *et al.*, 2005; Obara *et al.*, 2005),

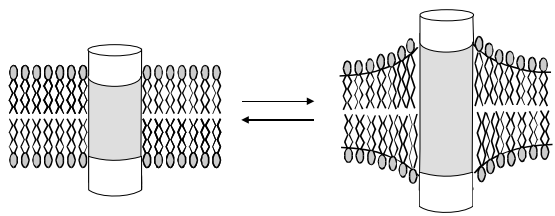

FIGURE 11 Possible effects of stored curvature elastic energy on the function of membrane proteins. The presence of lipids favoring the hexagonal H_{II} phase shifts the conformational equilibrium of an intrinsic membrane protein toward the conformation with the greatest hydrophobic thickness.

it is clear that the different conformations all have very similar hydrophobic thicknesses and yet the activity of the Ca^{2+}-ATPase is affected by the presence of phosphatidylethanolamine (Starling *et al.*, 1996a). The presence of phosphatidylethanolamine has been shown to affect channel opening of MscL (Moe and Blount, 2005) and, as described later, the hydrophobic thickness of MscL probably decreases on channel opening. However, even for MscL molecular dynamics simulations show different patterns of hydrogen bonding between MscL and phosphatidylethanolamines and phosphatidylcholines, associated with significant conformational changes on MscL (Elmore and Dougherty, 2003) that could be the underlying reason for the effects of phosphatidylethanolamine on MscL function.

E. Elastic Strain and Pressure Profiles

At equilibrium in a bilayer, hydrophobic forces exactly balance the repulsive lateral pressures present in the bilayer, and there is no net tension in the membrane (Seddon, 1990; Marsh, 1996). These forces are illustrated in Fig. 12. At about the position of the glycerol backbone region, just below the lipid headgroups, an attractive force F_γ arises from the unfavorable contact of the hydrocarbon chains with water (the hydrophobic effect). Tight packing in this region ensures the minimum exposure of the hydrocarbon interior of the membrane to water, leading to a negative lateral pressure (a positive membrane tension), tending to contract the bilayer. In the headgroup region of the bilayer, a positive lateral pressure F_h arises because of steric, hydrational, and electrostatic effects; these will normally be repulsive but may contain attractive contributions from, for example, hydrogen bonding interactions. Similarly, in the hydrocarbon interior of the membrane, attractive van der Waals interactions between the chains will be opposed by the repulsive interactions due to the thermal motions of the chains, the net effect being a positive lateral pressure F_c tending to expand the membrane.

For a membrane to stay flat, the forces illustrated in Fig. 12 must be in balance across the monolayer. We can say that a negative pressure in the glycerol backbone region, arising from the hydrophobic effect and serving to contract the membrane, balances a positive pressure in the chain region, serving to expand the membrane. Since the positive pressure in the chain region arises from collisions between the chains, and since the extent of chain motion varies down the chain as shown by the order parameter profiles shown in Fig. 6, different positions in the chains will make different contributions to this positive pressure. This has given rise to the idea of a pressure profile within the lipid bilayer. Pressure is an inherently macroscopic property, and so the concept of a local pressure is not absolutely straightforward (Lindahl and Edholm, 2000).

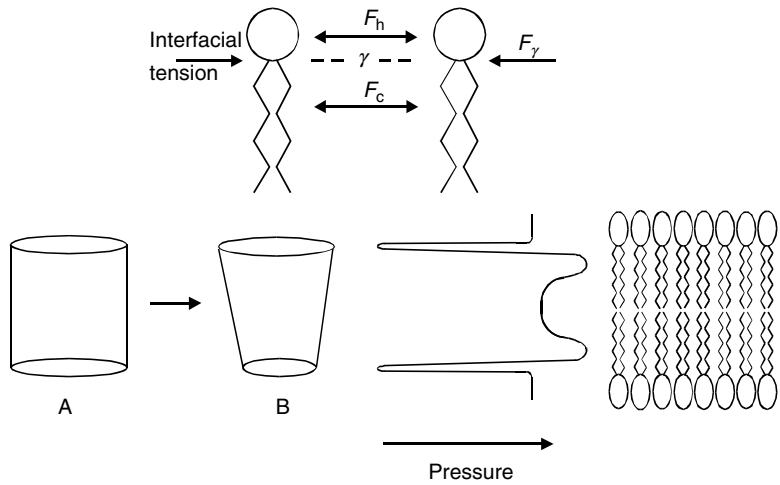

FIGURE 12 Pressure profile in a lipid bilayer. At the top is shown the distribution of lateral pressures and tensions across a lipid monolayer. The repulsive lateral pressure F_c in the chain region is due to thermally activated bond rotational motion. The interfacial tension γ, tending to minimize the interfacial area, arises from the hydrophobic effect (unfavorable hydrocarbon-water contacts). Finally, the lateral pressure F_h in the headgroup region arises from steric, hydrational, and electrostatic effects; it is normally repulsive, but may contain attractive contributions from, for example, hydrogen bonding interactions. After Seddon (1990). Below is the pressure profile in a lipid bilayer that will affect the conformational change A $\rightleftharpoons$ B for a membrane protein that involves a change in shape for the protein.

Nevertheless, pressure profiles can be calculated from molecular dynamics simulations by dividing the membrane up into a series of slices and calculating the pressures in each slice from the interactions between the atoms in that slice (Lindahl and Edholm, 2000; Gullingsrud and Schulten, 2004). As expected and as shown in Fig. 12, the distribution of lateral pressures within the fatty acyl chain region of a bilayer is not uniform (Cantor, 1999; Lindahl and Edholm, 2000; Gullingsrud and Schulten, 2003, 2004). The largest negative pressure occurs close to the backbone region of the bilayer, with the pressure of lowest magnitude in the center of the bilayer, corresponding to the region of lowest chain order parameter and lowest packing density where the ends of the chains from the two monolayers meet. The exact shape of the pressure profile was found to vary markedly with lipid structure (Cantor, 1999; Gullingsrud and Schulten, 2004). Cantor (1997) has suggested that this pressure profile could be important for membrane protein function.

In classical physical chemistry, to increase the volume of an object under constant pressure requires work, the work term being $P\Delta V$ where P is the applied pressure and ΔV is the increase in volume. In a lipid bilayer, because

there is no net tension (all the pressures in the membrane cancel out), simple expansion of a membrane protein with no change in shape requires no work. However, if a conformational change $A \rightleftarrows B$ for a membrane protein involves a change in shape in the transmembrane region of the protein, with the cross-sectional area of the protein at one depth in the membrane changing by more than the cross-sectional area at another depth, as illustrated in Fig. 12, then for the conformational change to occur, work will have to be done against the pressure profile since the $P\Delta V$ terms across the bilayer will not cancel. These energy terms could be large because the pressures involved have been estimated to be hundreds of atmospheres, that near the backbone region, for example, being estimated to be ca. 1000 atm (Cantor, 1999; Gullingsrud and Schulten, 2004). However, the calculations assume that the pressure profile of the lipid bilayer does not change as a result of the presence of the membrane protein or as a result of the change in shape of the membrane protein, and this seems unlikely since a key feature of the lipid bilayer is its plasticity, as illustrated in Fig. 7. A high degree of plasticity in the lipid bilayer means that the lipid bilayer will be able easily to distort around a membrane protein and so will provide little in the way of an energy barrier to changes in local volume of the type illustrated in Fig. 12.

Given that pressure profiles across lipid bilayers are predicted to change markedly with changes in the degree of unsaturation of the fatty acyl chains (Cantor, 1999), an experimental test for the importance of the pressure profile is to look for a marked dependence of protein function on the pattern of fatty acyl chain unsaturation. In fact, experiments in which Ca^{2+}-ATPase was reconstituted into a series of phosphatidylcholines containing fatty acyl chains of the same length but containing different numbers of *cis* double bonds showed no dependence of activity on chain structure, as long as the chain length remained constant (East *et al.*, 1984).

F. General Features of Lipid–Protein Interactions

A key feature of the lipid bilayer is its plasticity, allowing it to accommodate to the rough surface of a membrane protein and to accommodate readily those changes in membrane protein shape that are involved in function. The adaptations of the lipid bilayer required to accommodate a membrane protein are largely confined to the annular shell of lipid molecules surrounding the membrane protein (Lee, 1977) and, at least for some membrane proteins, activity is normal at low molar ratios of lipid to protein when only annular lipid is present (Warren *et al.*, 1974). Exchange between annular and bulk lipids is generally fast (East *et al.*, 1985), and selectivity in interactions between annular lipid and the transmembrane surface of a

membrane protein is correspondingly generally low (Lee, 2003). However, regions of high positive charge on a membrane protein close to the lipid headgroup region are likely to give rise to "hot-spots" where anionic lipids could bind with higher affinity than zwitterionic lipids (Marius *et al.*, 2005; Powl *et al.*, 2005a; Lee, 2006).

V. WHAT DO THESE GENERAL PRINCIPLES TELL US ABOUT MscL?

We would expect hydrophobic matching between MscL and the surrounding lipid bilayer to be highly efficient. This appears to be the case; Trp residues introduced at the ends of the second transmembrane α-helix of MscL from *Mycobacterium tuberculosis* maintain their positions close to the glycerol backbone region of the lipid bilayer when the lipid fatty acyl chains are changed in length from C12 to C24 (Powl *et al.*, 2005b). The hydrophobic thickness of MscL in the closed state has been estimated from Trp scanning fluorescence studies to be 26 Å (Powl *et al.*, 2005b), in good agreement with theoretical calculations based on the energetics of solvation of the protein in a model lipid bilayer (Lomize *et al.*, 2006).

Relative lipid-binding constants for MscL have been determined using a fluorescence quenching method, measuring the level of fluorescence quenching of Trp-containing mutants of MscL in mixtures of phospholipids with bromine-containing fatty acyl chains and non-bromine-containing fatty acyl chains (Powl *et al.*, 2003). As shown in Fig. 13, the phosphatidylcholine that bound most strongly to MscL was di(C16:1)PC, a lipid which forms a bilayer with a hydrophobic thickness of ca. 24 Å (Powl *et al.*, 2003), in good agreement with a hydrophobic thickness for MscL of 26 Å (Powl *et al.*, 2005b). The hydrophobic thickness of the native *M. tuberculosis* membrane will be close to 24 Å since the predominant lipid fatty acyl chains in the membrane are C18:1 and C16:0 (Coren, 1984).

As shown in Fig. 13, the chain length dependence of lipid binding to MscL in the closed state is less marked than that for the β-barrel protein OmpF (O'Keeffe *et al.*, 2000). β-Barrel proteins are relatively rigid so that, at least for moderate degrees of hydrophobic mismatch, hydrophobic matching can be expected to follow from distortion of the lipid bilayer around a relatively undistorted β-barrel (Lee, 2004). The fact that the variation in lipid-binding constant with chain length is less for MscL than for OmpF, therefore, suggests that hydrophobic matching for MscL is achieved by distorting both the protein and the lipid bilayer because it is less costly to distort an α-helical membrane protein than a β-barrel membrane protein (Powl *et al.*, 2003). Distortion of an α-helical membrane protein is likely to involve tilting of the transmembrane α-helices, as illustrated in Fig. 8.

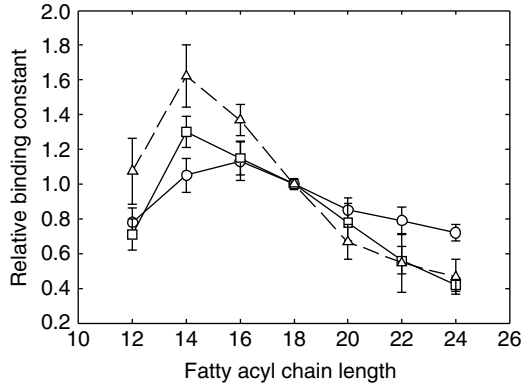

FIGURE 13 Relative lipid-binding constants for MscL. Binding constants for phosphatidylcholines relative to that for di(C18:1)PC are plotted as a function of acyl chain length for the closed channel (○) and for a gain of function mutant (□). Data are compared with relative lipid-binding constants for OmpF (Δ). Data from O'Keeffe *et al.* (2000), Powl *et al.* (2003), and Powl and Lee (2006).

To investigate lipid binding to the open state of the MscL channel, a gain-of-function mutant was prepared (V21K) in which Val21, at the narrowest region of the channel, was mutated to Lys, where charge repulsion is expected to keep the channel in an open state (Powl *et al.*, 2005a). The lipid binding most strongly to the V21K mutant is di(C14:1)PC (Fig. 13), a lipid giving a bilayer of hydrophobic thickness of ca. 20 Å, suggesting that channel opening is associated with a ca. 4-Å thinning of the lipid bilayer. This is consistent with the observation that decreasing bilayer thickness leads to a decrease in the tension required to open the MscL channel (Perozo *et al.*, 2002b). A thinning of 4 Å would be consistent with a ca. 16° increase in tilt angle for the transmembrane α-helices, in good agreement with the estimate made by Perozo *et al.* (2002a) for the *E. coli* protein.

The effect of lipid headgroup structure on the strength of lipid binding to MscL was investigated separately on the two sides of the membrane by introducing Trp residues on either the periplasmic or cytoplasmic sides of MscL (Powl *et al.*, 2005a). These studies showed that there was no selectivity in binding lipid of different classes on the periplasmic side of the membrane whereas on the cytoplasmic side, although phosphatidylcholines bound with equal affinity to phosphatidylethanolamines, anionic lipids, particularly phosphatidic acid, bound with high affinity to a site consisting of the three positively charged residues Arg98, Lys99, and Lys100 (Powl *et al.*, 2005a). These positively charged residues, together with adjacent negatively charged residues Glu102 and Glu104, were suggested to act as a molecular "Velcro,"

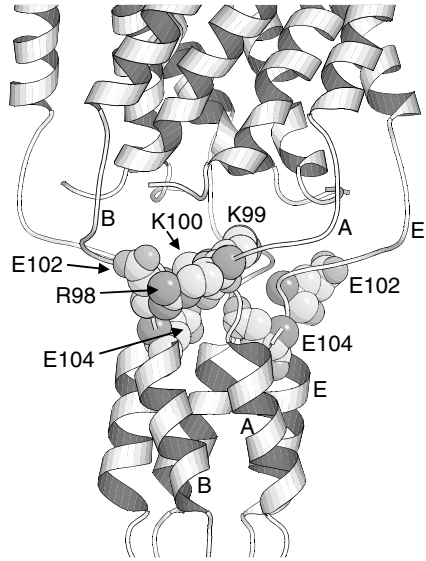

FIGURE 14 The location of the positively charged cluster Arg98, Lys99, and Lys100 in MscL at the C-terminal end of TM2 of the MscL monomer. The locations of Arg98, Lys99, and Lys100 in the central monomer (A) of the homopentameric structure are shown surrounded by the anionic residues Glu102 and Glu104 in the neighboring subunits B and E. The side chain of Lys100 is not resolved in the crystal structure.

holding together the ends of the TM helices in the closed form of the channel (Fig. 14). Opening the channel by helix tilting would move the ends of the helices further apart, only possible if the charge interactions observed in the closed channel were broken up. Indeed, simultaneous mutation of the three positively charged residues led to formation of the open channel (Powl *et al.*, 2005a). In the V21K open state mutant, the specific binding of anionic lipid was lost, showing that in the open state of the channel the positively charged cluster must have been repositioned so that high affinity interaction with anionic lipid headgroups is no longer possible (Powl *et al.*, 2005a).

The presence of a cluster of positively and negatively charged residues appears to be a characteristic of all MscL channels, although, given that the positively and negatively charged residues are located in a loop region of the structure (Fig. 14), it would not be expected that the positions of the charged residues would be absolutely conserved in the MscL sequences. Deletion of the charge cluster in the C-terminal region of *E. coli* MscL leads to loss of function, providing additional evidence that this region of MscL has an important role in channel function (Blount *et al.*, 1996). On the basis of

results obtained with a set of charge reversal mutants, Kloda *et al.* (2006) suggested that the charge cluster could act as a pH sensor in *E. coli* MscL.

Interaction of anionic lipid with the positively charged cluster on MscL seems not to be functionally important since Moe and Blount (2005) found that the tension required to open the MscL channel from *E. coli* was unaffected by the presence of anionic lipid. In contrast, Moe and Blount (2005) found that the presence of phosphatidylethanolamine increased the tension required to open the MscL channel, even though we found no selectivity for binding phosphatidylethanolamine over phosphatidylcholine, on either side of the membrane (Powl *et al.*, 2005a). Molecular dynamics simulations of MscL in bilayers of phosphatidylcholine and phosphatidylethanolamine suggest that the different patterns of hydrogen bonding to the phosphatidylcholine and phosphatidylethanolamine headgroups led to changes in protein structure (Elmore and Dougherty, 2003), and it is possible that these were responsible for the observed changes in tension for channel opening.

Trp fluorescence spectroscopy has also been used to investigate the role of lipid in maintaining MscL in the closed state. Wiggins and Phillips (2004) proposed that the closed state of the channel is stabilized by hydrophobic mismatch between the channel and the surrounding lipid bilayer and that energy differences between the open and closed states of the channel are dominated by bilayer deformation energies rather than by energy differences between the different conformational states of the protein itself. A Trp residue introduced into position 80 in the mutant F80W shows fluorescence emission centered at ca. 321 nm in the closed channel, but emission shifts to ca. 332 nm in the open mutant F80W:V21K (Powl *et al.*, 2005a). In a wide variety of detergents, fluorescence emission for F80W remained at ca. 321 nm, suggesting that a lipid bilayer is not required to maintain the MscL channel in the closed state and that the closed state of the channel is the most stable state for the wild-type protein (Powl and Lee, 2006). The driving force for channel opening would then be the less tight packing of the lipid molecules in the stretched membrane, leading to potential exposure of hydrophobic residues in the transmembrane α-helices to water, an exposure that can be minimized by expansion of the membrane protein to maintain good contact with the surrounding lipid bilayer.

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