CHAPTER 8

MscL: The Bacterial *M*echanosensitive Channel of *L*arge Conductance

Paul Blount, Irene Iscla, Paul C. Moe, and Yuezhou Li
Department of Physiology, University of Texas-Southwestern Medical Center, Texas 75390

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I. OVERVIEW

MscL is *m*echano*s*ensitive (MS) *c*hannel of *l*arge conductance found in the bacterial cytoplasmic membrane. It, with other MS channels, serves as a biological ''emergency release valve'' that protects the cell from lysis resulting from acute downward shifts in the osmotic environment. To date, more is known of MscL and its mechanism of action than perhaps any other MS channel. Many genetic and other functional studies have been performed on the *Escherichia coli* channel and a crystal structure exists for a closed or ''nearly closed'' structure of one of its orthologues from *Mycobacterium tuberculosis*. Models for the mechanisms of channel gating and the open structure have been generated and tested by several diverse approaches. Finally, several studies have begun to determine the precise stimuli that are sensed by this channel. Together, the data and projected models are giving a glimpse to the molecular mechanisms underlying an MS channel activity.

II. INTRODUCTION AND HISTORICAL PERSPECTIVE

Bacterial MS channels are not only a means to study aspects of bacterial physiology but are also currently the most advanced model system for studying MS channel function. They have thus emerged as a paradigm for studying how a protein can sense and respond to changes in its lipid environment. Among the bacterial MS channels, MscL has been the most tractable and is currently the best studied. Identification of the gene that encodes the MscL activity gave us a first glimpse and chance for the genetic study of a channel that senses and responds to mechanical force.

A. The Discovery of MS Channels in Bacteria

On genesis of the patch-clamp technique (Hamill *et al.*, 1981), it was just a matter of time before membranes that were not associated with electrical or other conductances, such as those belonging to cellular internal structures and organelles, were studied. Perhaps one of the more creative candidates was the cytoplasmic membrane of microbes, including bacteria. Ching Kung's group, the pioneer in the study of the physiology of microbial channels, was the first to patch the bacterial envelope of *E. coli* (Martinac *et al.*, 1987). To provide enough membrane area to be patched, the authors used cephalexin, a drug that inhibits septation; hence, the cells grew but could not divide. The resulting elongated cells were then collapsed by treatment with lysozyme and EDTA to form giant cells, on the order of a few microns in diameter,

which were then amenable to the patch-clamp technique. On examination, what were discovered were not voltage-gated channels, but rather those that responded to mechanical force, specifically suction or pressure within the electrode. These channels are most frequently referred to as MS channels, and as discussed in Section V.B have been shown to be gated by membrane tension. Remembering that *E. coli* is a Gram-negative bacterium, it was important to know which membrane, inner or outer, the channels resided in; subsequent studies demonstrated that the activities are not porins or other outer membrane channels but instead are restricted to the inner membrane (Berrier *et al.*, 1989).

B. Proposing a Function

A curious feature of these channels was that they had a relatively large conductance, on the order of 1 nanoSiemen (nS), whereas normal eukaryotic channels are at best measured in a few tens of picoSiemens. This observation begged the question of what function such a large MS channel activity could have in such a small cell. Previous studies had demonstrated that bacterial cells, when exposed to a high osmotic environment, accumulated compatible solutes such as K^+, proline, and glycine betaine to very high levels, presumably as osmoprotectants that would allow the cells to maintain turgor and continue to divide (Britten and McClure, 1962). When a culture so treated was subsequently subjected to an acute decrease in the osmotic environment, an osmotic downshock, the cells jettisoned the accumulated solutes to the medium without a substantial decrease in viability (Britten and McClure, 1962; Schleyer *et al.*, 1993). It seemed logical, therefore, to propose that this large-conductance channel is the conduit through which accumulated compatible solutes are expelled on osmotic downshock. However, as discussed in Section II.C, the demonstration that bacterial MS channels played a role as a "biological emergency-release-valve" required the cloning and characterization of multiple bacterial MS channels.

C. The Identification of Multiple MS Channel Activities in E. coli

The initial studies of bacterial MS channels (Martinac *et al.*, 1987, 1990) examined what was thought to be a single activity that was ~1 nS in conductance. In a subsequent study, it was demonstrated that when a larger stimulus was given, a larger conductance channel activity was observed; the two activities could be separated by biochemical fractionation of solubilized membrane proteins over a gel filtration column and reconstitution of the

subsequent fractions into azolectin lipids, which were subsequently subjected to patch clamp (Sukharev *et al.*, 1993), thus demonstrating that the MscS and MscL channel activities were not only produced by separate proteinatious entities but that they survived solubilization, biochemical enrichment, and reconstitution. An additional study showed that with smaller stimuli a smaller conductance was also observed (Berrier *et al.*, 1996). The gradation of channel activities with increasing conductance correlating with an increase in stimuli to gate them thus led to the hypothesis that as osmotic downshock increased, more and larger cytoplasmic components would be jettisoned to the medium to prevent cell lysis (Berrier *et al.*, 1996). From these studies, a new nomenclature emerged, with the largest conducting channel (3.6 nS) named MscL for MS *c*hannel of *l*arge conductance, MscS for the *s*maller conducting channel (1 nS), and MscM for the even smaller or *m*ini conducting channel (0.3 nS). Although the molecular identity of MscM has remained elusive, the other channels have been cloned and sequenced (see Section II.D for a discussion of the cloning of the MscL channel). The final added twist of the story came when it was realized that what had been deemed "MscS activity" was probably composed of two similar channels, now referred to as MscS and MscK; "K" for K^+ regulated (Levina *et al.*, 1999; Li *et al.*, 2002). From what we now know of the abundance and conductance of the different channel activities, it appears that the early studies characterizing MS channels in *E. coli* were characterizing primarily the MscS, and perhaps a small amount of the MscK activity. Please note that the *E. coli* MscS channel is reviewed in Chapter 9 and MscS/MscK related putative channels found within plants reviewed in Chapter 13 of this volume.

As discussed in Section II.B, the role of these channels was suspected to be as biological emergency-release-valves that allow the cell to rapidly adapt to an acute osmotic downshock. However, the discovery of multiple channel activities left open the possibility that these channels would be redundant in function. Indeed, it required the cloning of both the *mscS* and the *mscL* genes, and the generation of a double null to definitively demonstrate that these channels played such a role (Levina *et al.*, 1999). However, making a triple-null strain that, in addition, is null for *mscK* does not amplify the "osmotic-lysis" phenotype observed for double-null mutant. MscK is one of a handful of MscS homologues predicted to be expressed in *E. coli* but is the sole homologue to be observed in patch clamp. MscK is only observed under specific ionic environments, thus leading to the hypothesis that some MscS homologues may only function under as yet unidentified environmental conditions (Li *et al.*, 2002). Of all of the bacterial channels, the gene encoding MscL was the first to be identified. Although initially the isolation of this single gene could not confirm the physiological function of the bacterial channels because of the redundancy of function with the, at the

time, unidentified MscS, it did reflect an important step in the field of bacterial mechanosensors thus allowing for the study of the molecular basis of mechanosensation in a well-defined system.

D. Identification of the E. coli mscL *Gene*

The identification of the *E. coli mscL* gene was performed by a laborious effort to first biochemically enrich the protein responsible for the MscL activity (Sukharev *et al.*, 1994). To our knowledge, this approach had not been used previously, or since, for the molecular identification of a channel. Briefly, membrane fractions were solubilized, fractionated using columns that separated the proteins according to biochemical properties, then a portion of each fraction reconstituted into azolectin lipids and assayed by patch clamp for channel activity. Fractions containing a significant number of channels were pooled, assayed for protein content by SDS-PAGE, and then chromatographically fractionated over a second, independent column. This was performed until SDS-PAGE showed only one primary protein band remained, thus suggesting that the channel was generated from a single gene product. This 17-kDa band was sequenced, and 37 amino acids at the N-terminal identified. At the time, the *E. coli* genomic sequence was just under way and the sequence did not match any of the genome-project sequences. But, the authors were lucky because researchers studying another gene, *trkA*, had sequenced slightly further than necessary, thus generating a sequence that predicted the first 38 residues of the next gene (Hamann *et al.*, 1987). This was a perfect match with the putative MscL microsequence. Thus, this information identified the region of the genome that held the proposed *mscL* gene and allowed for its cloning and sequencing (Sukharev *et al.*, 1994). Surprisingly, the gene was very small, encoding a protein of 136 amino acids in length and only two α-helical transmembrane domains. The discrepancy between the size of the protein on SDS-PAGE and in a nondenaturing gel filtration column strongly suggested a homomultimer.

The authors of this first report of the identification of *mscL* went to great lengths to demonstrate that they had indeed cloned the gene responsible for the MscL activity (Sukharev *et al.*, 1994). An *mscL*-null mutant was generated by insertional disruption; to demonstrate that the channel activity was missing from this null mutant, 50 spheroplast membrane patches that survived a high level of stimulus were shown not to contain activity. Expression of *mscL* in *trans* in the null mutant reconstituted the activity. The SDS-PAGE protein band that initially correlated with MscL activity was missing from the *mscL*-null mutant on an identical enrichment scheme used to identify it, and no activity could be reconstituted from the final fraction

when assayed by patch clamp. Finally, when the gene was translated using a cell-free system and the product reconstituted and assayed by patch clamp, MscL channel activity was observed. At the time, little was known of the molecular basis of mechanosensation. Perhaps the best-studied mechanosensory system at the time was in *Caenorhabditis elegans* where several genes were found to correlate with touch sensation and the few that predicted transmembrane proteins were candidates for channel subunits (Tavernarakis and Driscoll, 1997); however, there was no gene, or combination of genes, shown to encode MS channel activity. Even today, the precise functional role of many of the candidates for eukaryotic MS channels is still being debated. Hence, *mscL* underwent a rare set of rigorous tests to demonstrate that it truly encoded the MscL activity; as a result, it was the first gene shown to encode any MS channel activity.

E. Early Mutagenesis Studies

As discussed in Section II.B, the proposed function of bacterial MS channels was as biological emergency-release-valves that allowed the cell to rapidly adapt to an acute osmotic downshock. However, after the cloning of *mscL*, it soon became clear that the MscL-null mutant did not have an obvious phenotype; it did not lyse or show any distress on osmotic downshock. Hence, the early mutagenic studies of MscL served two purposes: first to determine the functional significance of domains and residues within the protein, and second, to strengthen the correlation between osmotic and "compatible solute-flux" phenotypes and the functional properties of the channel activities.

The earliest mutagenic studies were largely directed at determining functional regions of the protein. Deletion analysis demonstrated that much of the C-terminal region was not necessary for normal channel function, while deletion or alteration of the N-terminal led to a disruption of channel function (Blount *et al.*, 1996a,c; Häse *et al.*, 1997a). In addition, site-directed mutations within the channel often led to changes in channel sensitivity to pressure or open dwell times (Blount *et al.*, 1996c).

While site-directed mutagenesis can, and did, give a gross resolution of some regions of the protein that did or did not play a role in MS channel activity, it was not overlooked that one of the real advantages of working with a bacterial system is the ability to randomly mutate a gene of interest and select or screen for rare phenotype-effecting mutations. However, as stated above, the *mscL*-null mutant did not show an obvious loss-of-function (LOF) or null phenotype, thus limiting the possibility of isolating mutated genes that led to total or partial LOF phenotype. Ching Kung's group therefore isolated randomly mutated *mscL* genes that, when expressed, led

to a slowed- or no-growth gain-of-function (GOF) phenotype (Ou *et al.*, 1998). The approach was to place the randomly mutated *mscL* genes under the transcriptional control of an inducible promoter (lacUV5) and use a replica-plate strategy; colonies that grew when the gene was not induced, but failed to grow well when the channel expression was induced, were selected for further characterization. The hope was that a subset of mutations would lead to channels that mis-gate *in vivo*; this "loose cannon" would cause the cells to inappropriately lose valuable cytoplasmic components and, therefore, play "metabolic catch-up" and either slow their growth or even decrease their viability. This study led to two major findings. First, it identified a "hot-spot" within the protein where specific mutations led to extremely severe GOF phenotypes; this region was predicted to be the N-terminal half of the first transmembrane domain (TMD1). Second, further analysis of the functional properties of the mutated channels demonstrated a correlation between the severity of the phenotype, the flux of the osmoprotectant K^+, and an increase in sensitivity of the channel activity to stimuli. Most of the mutations in this hot-spot were hydrophilic residues, and many were to charged amino acids. Furthermore, in addition to an increase in channel sensitivity, the authors noted that the GOF mutants also showed a decrease in the open dwell time, suggesting that the transition barrier between closed and open states was decreased. However, a more complete appreciation of these findings required a more detailed structural model.

III. A DETAILED STRUCTURAL MODEL: AN X-RAY CRYSTALLOGRAPHIC STRUCTURE FROM AN *E. COLI* MscL ORTHOLOGUE

Subsequent to the identification of *mscL*, the topology of the MscL protein had been well defined (Blount *et al.*, 1996b) using an approach common for bacterial systems, PhoA fusion (Manoil and Beckwith, 1986). This study supported the hypothesis that the protein had two transmembrane domains, and strongly suggested that the both N- and C-termini were cytoplasmic and the loop between the transmembrane domains periplasmic (Blount *et al.*, 1996b). By contrast, other data were misleading and misinterpreted to suggest that the complex was composed of six identical subunits (Blount *et al.*, 1996b; Saint *et al.*, 1998), rather than the five we now know it contains. Finally, one study using circular dichroism (CD) spectral analysis demonstrated that the protein was highly α-helical and relatively resistant to denaturation (Arkin *et al.*, 1998). This was essentially the extent of the knowledge of the structural features of the channel when an X-ray crystallographic structure of an MscL from *M. tuberculosis* was obtained and revolutionized the field (Chang *et al.*, 1998). Because most experimental data has

been derived from the MscL from *E. coli*, researchers have been forced to make structural and functional comparisons between these orthologues.

A. The Crystal Structure

Douglas Rees's group revolutionized the field of bacterial MS channels when they solved a structure of an MscL by X-ray crystallography to a resolution of 3.5 Å (Chang *et al.*, 1998) (Fig. 1). At the time, because of the numerous microbial genomic sequencing projects, it was becoming obvious that MscL is almost ubiquitous among the prokaryotic kingdom and that large portions of the protein are highly conserved (Moe *et al.*, 1998; Maurer *et al.*, 2000). The authors, therefore, apparently used the strategy of attempting to generate refracting crystals from homologues derived from various organisms. Hence, the solved structure was derived from the organism *M. tuberculosis*. (For those readers that would like a more detailed discussion of the structural properties of MscL and MscS than that presented within this chapter, below, please refer to Chapter 1 of this volume.)

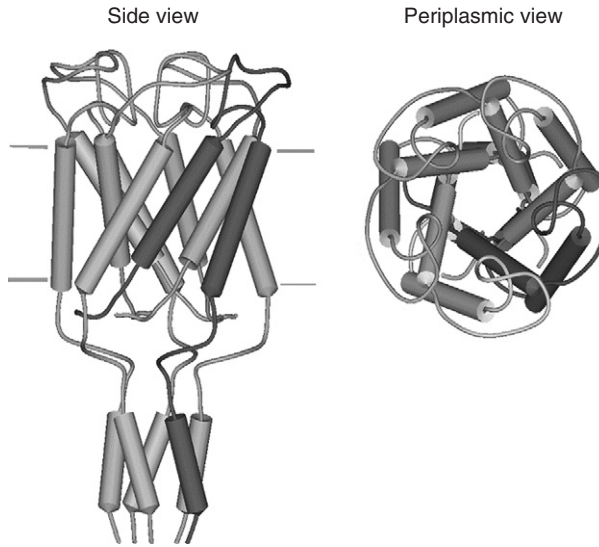


FIGURE 1 Schematic representation of MscL based on the *M. tuberculosis* crystal structure, which some evidence now suggests is in a "nearly closed" state. In the view from the side, the approximate position of the membrane is shown (left). The disposition of the TMD1 lining the pore of the channel and the TMD2 surrounding them is better shown in the periplasmic view of the protein (right). A single subunit is highlighted for clarity.

The solved structure confirmed some predictions, challenged others, and shed new light on structural features for which no data existed. As had been anticipated from previous studies (Blount *et al.*, 1996b; Arkin *et al.*, 1998), the structure is highly α-helical and contains what appear to be two transmembrane domains. However, the structure revealed a radially symmetrical homopentamer in contrast to the hexamer (Blount *et al.*, 1996b; Saint *et al.*, 1998) or even monomer (Häse *et al.*, 1997b) that had been predicted from previous studies. Within the crystallographic study, a cross-linking experiment supported the pentameric structure for both the *M. tuberculosis* (Tb-MscL) and *E. coli* MscL (Eco-MscL). A subsequent study that utilized multiple cross-linking reagents as well as equilibrium ultracentrifugation further supported the pentameric design of the MscL channel (Sukharev *et al.*, 1999a), which is now generally accepted. The C-terminal region of the channel appeared to form a cytoplasmic α-helical bundle, which the authors noted may be an artifact of the low pH in which the channel was crystallized. Although deletion experiments suggested that the N-terminus was important in forming a functional channel, no clues came from the structure because the first nine residues were not resolved. The second transmembrane domain (TMD2) faced what would normally be the lipid bilayer, while the TMD1 formed the pore. The constriction point was just over 4 Å in diameter; so, given that the conductance of the channel and molecular sieving experiments predicted the open channel to form a pore greater than 30 Å, the authors speculated that the channel was in a closed or "nearly closed" state.

There was nothing obvious from the structure to reveal the functional working of an MS channel. Hence, mechanistic models required a combination of the solved structure with data that had been derived from the earlier mutagenesis studies.

B. Fitting the Structure with the Findings from Mutagenesis Studies

The crystallographic structure of Tb-MscL showed the constriction of the pore of the channel being formed by TMD1, with the periplasmic half of the domain forming a vestibule, while the cytoplasmic half appeared more tightly packed with a true constriction point at a valine at position 21 (V21; the analogous residue is V23 in *E. coli*) (Chang *et al.*, 1998). The authors noted this "hydrophobic barrier or gate" at the cytoplasmic half of the TMD1, and discussed the consistency with the random mutagenesis study (Ou *et al.*, 1998) that found this region to be a hot-spot, with many mutations in this region leading to GOF phenotypes (Section II.E). A closer look at the mutagenesis data showed that 14 of the 18 GOF phenotype-effecting mutations isolated were within this subdomain, all but 3 of the 14 were

to residues that were significantly more hydrophilic, 7 of which were to charged residues. In addition, it was noted that there was a correlation between an increase in severity of the GOF phenotype *in vivo* with both, an increase in sensitivity of the channel to membrane tension and a decrease in channel open dwell times as assayed in patch clamp (Ou *et al.*, 1998). This correlation was subsequently further substantiated by two studies: one in which multiple residue substitutions at a single position, G22 (Yoshimura *et al.*, 1999), and another in which it was shown that adding a charge to the channel posttranslationally by reacting sulfhydryl reagents with a cysteine mutant (Yoshimura *et al.*, 2001). Combining these data with the structure led to what was coined the "hydrophobic lock" hypothesis (Blount and Moe, 1999; Yoshimura *et al.*, 1999; Moe *et al.*, 2000). Briefly, according to this theory, it is the transit of this hydrophobic constriction point of the pore through an aqueous environment, presumably the channel lumen, during a normal transition state that is the primary energy barrier to channel opening. The substitution of a residue in this region to a more hydrophilic amino acid decreases this energy barrier, thus allowing the channel to more easily transit from the closed to the open state, and back again. Thus, the lowering of the transition barrier leads to the increased sensitivity of the channel as well as the observed decrease in open dwell time.

C. Comparing Tb-MscL with Eco-MscL

Given that the best structural information is for Tb-MscL, but that the bulk of the experimental data has been obtained from the Eco-MscL, it is tempting to shoehorn all of the data obtained for Eco-MscL into the obtained structure. Aspects of the Tb-MscL structure have been confirmed for the Eco-MscL channel by electron paramagnetic resonance (EPR) spectroscopy in combination with site-directed spin labeling (SDSL), albeit to a far less precise resolution (Perozo *et al.*, 2001). However, one must be careful to take into account any functional differences between these potential orthologues, and to realize that assumptions that details of the structure are conserved, or that the structure reflects a fully closed state of the channel, may not be correct.

1. Functional Comparisons

One report had assayed seven homologues from both Gram-positive and Gram-negative organisms, when expressed in an MscL-null *E. coli* strain, and found that all of the putative channel genes encoded detectable channel activity (Moe *et al.*, 1998); hence, the channels all appeared to be orthologues. However, functional properties were not always identical. For example,

the channel isolated from *Synechocystis* was found to be significantly less sensitive to membrane tension and that isolated from *Staphylococcus aureus* was found to have significantly shorter open dwell times when compared to Eco-MscL. The report had two important implications. First, that the function was conserved; a phenomenon that would seem unlikely unless the channel activity that could be measured was a critical part of the protein's normal *in vivo* function. Second, that the most conserved parts of the protein among these and other homologues, for example TMD1, must have exceptional functional relevance. Unfortunately, the Tb-MscL was not among these initially characterized MscL channels.

Subsequent to the report of the crystallization of the Tb-MscL channel, a study was made of the functionality of this potential orthologue when expressed in an MscL-null *E. coli* strain (Moe *et al.*, 2000). Although channel activity was observed, the channel was among the least sensitive of all homologues assayed to date; the *Synechocystis* MscL is the only other channel that is this difficult to open. Consistent with this finding, the Tb-MscL, when expressed in *trans*, was unable to suppress the osmotic-lysis phenotype observed for the *mscS*/*mscL* double-null *E. coli* mutant. On the other hand, Eco-MscL mutations that had been shown to effect a GOF phenotype, when transposed into Tb-MscL, were also effective at making this channel more sensitive, suggesting a mechanistic correlation between the two channels (Moe *et al.*, 2000). Hence, the inability of the Tb-MscL channel to gate at physiological membrane tensions when expressed within *E. coli* may simply be due to environmental factors such as native membrane composition; this functional difference should be considered when trying to extrapolate data derived from the Eco-MscL to the structure of Tb-MscL.

2. Does the Structure Reflect a Fully Closed State?

The authors of the crystallization paper were extremely careful to state that the channel appeared to be in a closed or nearly closed state (Chang *et al.*, 1998). Perhaps because it was suspected that the closed state would be of the lowest energy, many researchers dogmatically believed that the Tb-MscL structure reflected a fully closed structure. However, relatively recent and independent lines of evidence have suggested that for the Eco-MscL channel G26, rather than V23, is the true constriction point of the fully closed channel. In a study in which the two transmembrane domains were sequentially scanned by cysteine mutagenesis, G26C, not V23C, appeared to efficiently make disulfide bridges; channel activity was only efficiently observed for the G26C mutant in patch clamp when DTT was added to the patch buffer (Levin and Blount, 2004). Furthermore, in an independent study, a number of single histidine mutations were generated in the pore region of Eco-MscL, and the ability of these residues to coordinate metals,

including Ni^{2+}, Cd^{2+}, and Zn^{2+}, was assayed as the ability of these metals to keep the channel closed or inhibit it from gating (Iscla *et al.*, 2004). Again, G26H, not V23H, was the most efficient at coordinating these metals, suggesting that this is indeed the constriction point. If true, this could simply be species specific. On the other hand, the Tb-MscL structure shows a channel with a greater than 4-Å pore. It, therefore, seems possible that for both channels the analogous and conserved glycine is closer to the constriction point. This would predict that the constriction point would be more periplasmic and the vestibule smaller. It would also require a counterclockwise rotation of TMD1 when viewed from the periplasm; since, as discussed in Section IV.A.2, a clockwise rotation of TMD1 has been predicted to occur on channel opening, such a counterclockwise rotation may be necessary to achieve a fully closed channel. Hence, the Tb-MscL structure may not reflect a truly fully closed but merely a nearly closed state of the channel.

IV. PROPOSED MODELS FOR HOW THE MscL CHANNEL OPENS

Given a crystal structure for a (nearly) closed state of a channel, it is tempting for researchers to speculate on what the open structure would look like. In the original crystallization paper, the authors included a crude model in which the TMD1s separated (Chang *et al.*, 1998). To form a pore of greater than 30 Å with near-vertical transmembrane helices, all 10 transmembrane domains must contribute to the channel lumen. More recently derived evidence strongly suggests that this simple model of the pore being generated by transmembrane domains positioned in the membrane like staves of a barrel is incorrect. Although the concrete evidence of a crystal structure for the open structure of the MscL channel is still lacking and much of the details are still in debate, from several independent studies an image of the open structure of MscL channel is beginning to emerge.

A. Opening the Channel: Twist and Turn

The first detailed model proposed for the open and transition states for MscL opening was both revolutionary and bold (Sukharev *et al.*, 2001a,b). It was revolutionary because it proposed a mechanism by which the lumen of the pore could be generated almost solely by the TMD1. This could be achieved by increasing the angle in which these domains sit within the membrane; the opening would then not be much different from the opening of the iris of a camera. The model was bold because it not only proposed a critical function as a second gate for the N-terminal end of the protein

(Sukharev *et al.*, 2001a), which was not resolved in the crystal structure (Chang *et al.*, 1998), but it also predicted the precise locations for each individual residue in the open and several transition states (Sukharev *et al.*, 2001b). The notion of the iris-like opening of the channel and TMD1 lining the pore can now be seen as a great insight that is generally agreed on by researchers in the field, and perhaps therefore often underappreciated. On the other hand, it is perhaps not surprising that many of the other details of the model are either highly controversial or largely discredited by the numerous subsequent studies.

1. Tilting the Transmembrane Domains

In proposing the detailed model, the authors chose to work with the Eco-MscL channel, presumably because of the wealth of mutagenic information and the ability to test the models on a channel that had a practical functionality. Therefore, before trying to derive the open structure, the authors generated a model for the closed Eco-MscL channel largely by imposing the sequence onto the Tb-MscL structure and making a best-guess for any discrepancies (Sukharev *et al.*, 2001b). From this model, predictions were made on how the Eco-MscL channel opens. One of the major predictions was that the transmembrane domains would tilt within the thinning membrane and then TMD1 would separate to open the channel. This would allow the lumen of the pore to be formed largely by TMD1. The resulting models for the structures for the closed and open states of the channel are shown in Fig. 2.

Several studies, using various approaches, have now provided strong support for the idea that the transmembrane domains tilt and TMD1 lines the lumen of the pore. In one study, lysophospholipids were used to gate the Eco-MscL channel *in vitro* and structural aspects of the open channel were determined by SDSL and EPR (Perozo *et al.*, 2002a). The findings were consistent with the tilting and expansion of the transmembrane domains, as predicted (Sukharev *et al.*, 2001b). In another study, Eco-MscL was divided in half so TMD1 and TMD2 were expressed independently (Park *et al.*, 2004). By patch-clamp analysis, the half-containing TMD1 formed spontaneously gating channels, albeit of varying conductance, while TMD2 segments were completely silent as assayed by patch clamp. The coexpression of the two domains formed an MS channel with a similar conductance to the wild-type channel. Hence, the data from this study suggested that TMD1 plays the large part in forming the channel lumen, while TMD2 is more important for assembly and sensing the lipid environment. In yet another study, tryptophan or tyrosine residues were substituted into strategic positions in the transmembrane domains in an attempt to decrease the ability of the domains to tilt (Chiang *et al.*, 2005). The results are consistent with gating of the channel

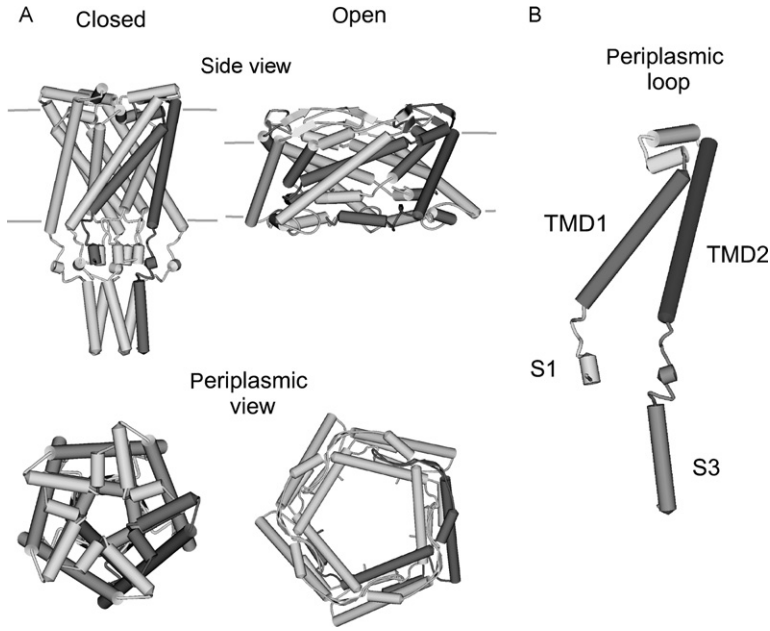


FIGURE 2 One proposed model for the structure of the *E. coli* MscL protein (Sukharev *et al.*, 2001b). (A) In the upper part of the panel, the lateral views of the homopentameric complex are shown in its open (right) and closed (left) states. The approximate position of the membrane is shown. Note the flattening of the open structure in comparison with the closed conformation due to the tilting of the transmembrane domains. Although the S3 domain is shown to separate, a more recent proposal suggests that the bundle may remain intact (Anishkin *et al.*, 2003). In the lower panels, the respective periplasmic views are shown. (B) The different domains of the protein are shown in a single subunit.

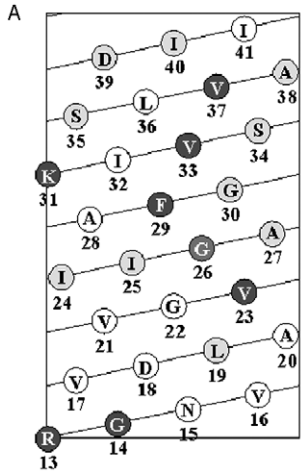
being linked with TMD tilt; however, specificity of the phenotypes to the substitutions was not demonstrated [e.g., a previous study (Levin and Blount, 2004) demonstrated that a C at position F93 yields a similar decreased-sensitivity phenotype as the F or W substitutions studied]. Finally, one study used an approach coined an *in vivo* SCAM (Bartlett *et al.*, 2004); SCAM for *s*ubstituted *c*ysteine *a*ccessibility *m*ethod (Akabas and Karlin, 1999). Here, a characterized cysteine Eco-MscL library in which each residue was independently mutated in both transmembrane elements (Levin and Blount, 2004) was used to determine the accessibility of each residue in the closed and open states of the channel. The authors, taking advantage of the fact that there are no endogenous cysteines in MscL, extended previous findings that the addition of hydrophilic residues in the pore often led to a channel that gave

a GOF phenotype (Ou *et al.*, 1998; Yoshimura *et al.*, 1999, 2001), that a cysteine substitution allowed for posttranslational modification of the residue by using sulfhydryl reagents (Akabas and Karlin, 1999), and that this approach had been successfully used for a single substitution previously (Batiza *et al.*, 2002). The positively charged sulfhydryl reagent methanethiosulfonate bromide (MTSET) was used to modify the cysteine mutations *in vivo*, and the assay was performed in the presence or absence of an osmotic downshock sufficient to gate the channel to assay accessibility in the open and closed states of the channel, respectively. A viability cell count was used to determine if the residue was accessible and modified the channel such that it inappropriately gated and thus effected a GOF phenotype. Patch-clamp analysis of these mutants in the presence and absence of MTSET has substantiated and extended these results (Bartlett *et al.*, 2006). Although a negative result in this assay may simply mean that the modification does not lead to a misgating channel, it is still of interest to note that all of the 11 residues that gave a positive result in this assay are in the TMD1 (Bartlett *et al.*, 2004). While none of the experiments above are as definitive as a crystal structure of the open channel, together they form a strong support for the original postulate that the transmembrane domains tilt and that TMD1 alone forms the bulk of the lumen of the pore.

2. Rotating TMD1

The original model for the open channel of Eco-MscL simply separated the TMD1 domains and predicted only a very small counterclockwise rotation of this domain of the channel when viewed from the periplasm (Sukharev *et al.*, 2001b). The model predicted several interactions, including unique interactions in the open state between TMD1 and TMD2, that were subsequently tested by trying to trap the channel in specific states by the generation of disulfide bonds in double-cysteine mutants (Betanzos *et al.*, 2002); this approach is often called disulfide trapping. However, it is important to note that the cysteine mutations were highly targeted so only very limited combinations of cysteine substitutions were tested. In addition, when disulfide trapping was reported to stabilize the open state of the channel, as determined by an increase in open dwell time of the fully open state in three patches, membrane tension was still required to open the channel (Betanzos *et al.*, 2002), and the observation that other mutations at independent locations within the channel have been reported to lead to such increases in open dwell times (Blount *et al.*, 1996c) makes the interpretation of the disulfide trapping slightly more difficult to interpret. Finally, a reversibility of the stabilization of the open state with reducing reagents was not shown.

In contrast to the above model, the model derived from SDSL and EPR suggested a 110° clockwise rotation of the TMD1 domain on gating (Perozo



Perozo *et al.*, 2002a	Sukharev *et al.*, 2001b
L19	R13
I24	G14
I25	V23
G26	G26
A27	F29
G30	K31
S34	V33
S35	V37
A38	
D39	
I40	
M42	

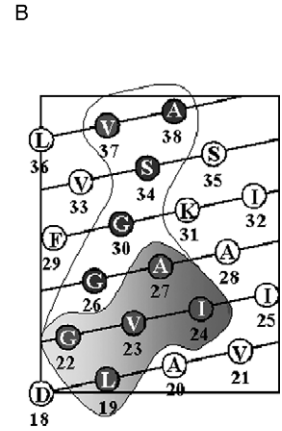


FIGURE 3 The residues predicted by different studies to line the pore region of *E. coli* MscL in its open state. (A) Helical net representation of residues R13 to I41 from the TMD1 (left). Residues predicted to be exposed to the pore in the open state of the channel by the original (Sukharev *et al.*, 2001b) model are shown in dark gray circles, while those residues derived from EPR studies (Perozo *et al.*, 2002a) are shown in light gray circles. Residue G26, the only one that is included by the both models, is shown in gray. A table listing the different set of residues lining the pore of the channel in the two models is shown (right). (B) Helical net representation of residues D18 to A38 where residues predicted to be exposed to the pore of the channel on activation are shown in light gray circles, as indicated by the results of an "*in vivo*" SCAM study (Bartlett *et al.*, 2004) (see text). Residues in dark gray circles were predicted to be exposed to the lumen in the closed state.

et al., 2002a). Hence, the discrepancy with the previously proposed model was close to a full 180°. A summary of the residues predicted to line the lumen of the open pore for the two models is presented in Fig. 3; note that G26 is the only common residue.

The results of the *in vivo* SCAM (Bartlett *et al.*, 2004), which identified residues exposed to the aqueous environment when the channel was in the closed and opening states as discussed in Section IV.A.1, appear to resolve which residues are exposed to the channel lumen (Fig. 3). Note that this approach, in contrast to those used previously, has the advantage that movements of the channel are assayed *in vivo*, when the channel is in its natural environment. Consistent with the hypothesis that G26 is the constriction point of the closed channel (Section III.C.2), G26C appeared to be highly reactive with MTSET in the closed state. The residue clockwise to this position, A27, however, required gating to observe maximal effects even though it is more periplasmic. Furthermore, the sequential residues G22,

V23, and I24 all required gating for maximal effects, and absolutely no effect was observed for I24 unless gating occurred. The only way I24 could efficiently be exposed to the lumen of the open pore would be if there was a significant clockwise rotation of the TMD1 domain. Hence, these data are consistent with the EPR results.

In another study, mentioned in Section III.C.2, a number of single histidine mutations were generated in the pore region, and the capacity of these residues to coordinate metals, including Ni^{2+}, Cd^{2+}, and Zn^{2+}, was tested by their ability to keep the channel closed or inhibit it from gating (Iscla *et al.*, 2004). Again, the I24 residue was one of the positions assayed. Each of the metals assayed was able to inhibit gating of the I24H mutant. These data not only support the hypothesis of a relatively large clockwise rotation of the TMD1 domain but also suggest that this rotation often occurs quite early in the gating process, even preceding ion permeation.

As discussed in Sections II.E, a random mutagenesis study identified a number of GOF-effecting mutations. In one study, two of these mutated channels, V23A and G26S, were further randomly mutated and suppressors of the GOF phenotype isolated (Li *et al.*, 2004). Partial suppressors were preferentially isolated to avoid nonfunctional channels. All of the suppressors isolated were found to be "general suppressors" that suppressed both GOF-effecting mutations, with one exception, I92V, which suppressed exclusively the G26S mutation. These data suggest a direct interaction between G26 and I92 on gating. When these positions are imposed on the original open-state model, they fall in proximity but do not face each other (Fig. 4). However, a simple rotation of close to 180° would do the trick. Here again, the data are consistent with a clockwise rotation of the TMD1 domain.

In sum, the original model for the open channel structure predicted a slight counterclockwise rotation of the TMD1 domain. Although some disulfide-trapping experiments have supported this model, an EPR study, the *in vivo* SCAM, metal binding to histidine substitutions, and suppressor mutagenesis all support a model in which there is a significant clockwise rotation of the TMD1 domain.

3. A Closed-Expanded State

An early study defining the energetic and spatial parameters of the gating of the Eco-MscL channel suggested an expansion of the system prior to channel gating (Sukharev *et al.*, 1999b). Assuming the expansion does not occur in the lipid membrane itself, this could be accounted for if the channel expanded to two-thirds its fully open size prior to gating. This begged the question: could there be expansion of the pore, but permeation barred by a second gate? One of the boldest proposals in the original gating models for the Eco-MscL channel was that the N-terminal region of the protein, which

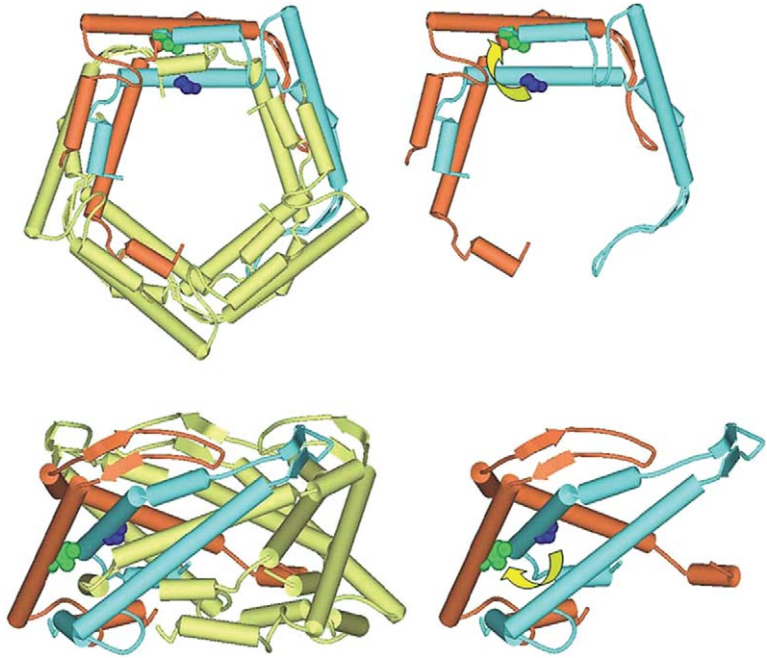


FIGURE 4 The MscL G26 and I92 residues may be in proximity. One study using a "suppressor mutagenesis" approach, as described in text, predicted an interaction between G26 and I92 (Li *et al.*, 2004). As seen in a current model for the open structure of the homo-pentameric *E. coli* MscL channel (Sukharev *et al.*, 2001b), G26, shown in CPK style in blue on the cyan subunit, and I92, in green on the adjacent orange subunit, are in relatively proximity. Cytoplasmic views (top) and side views (bottom) containing all five (left) or just the two noted adjacent subunits (right) are presented. Note that all is necessary for interaction of these residues is the rotation of TM1 (shown by the arrows in the right panels).

was not resolved in the Tb-MscL crystal structure, served as this second gate (Sukharev *et al.*, 2001a). The authors proposed that this region of the protein formed a helical bundle just cytoplasmic to the TMD1 pore. These proposed α-helical domains at the extreme N-terminal end were referred to as "S1 domains." The model predicts that the TMD1 pore could expand without ion permeation and stabilize in what was called the closed-expanded (CE) state. The model further predicted that it was the separation of the S1 bundles that actually allowed ions and other molecules to permeate. This model was appealing because it could be the sequential separation of the S1 segments that accounted for the substates of the channel that were often observed. In addition, previous studies had demonstrated the importance of this region

of the protein for channel function; deletion of the first 11 residues abolished all channel activity (Blount *et al.*, 1996a,c; Häse *et al.*, 1997a). Here again, targeted disulfide-trapping experiments were performed to support the model (Sukharev *et al.*, 2001a).

While appealing, the model cannot account for all of the data. First, previous studies had demonstrated that a substate that is four-fifths normal conductance is the one that is most often stabilized (Sukharev *et al.*, 1999b), which seems unlikely with three bundled cytoplasmic S1 helices as proposed. Second, as discussed in Section II.E, substitutions within TMD1 invariably lead to changes in the kinetic properties of the channel as well as increasing its sensitivity; these observations led to the hydrophobic lock hypothesis, a proposal that separation of the TMD1 domains is the primary energy barrier for gating (Blount and Moe, 1999; Yoshimura *et al.*, 1999; Moe *et al.*, 2000) (Section III.B). This change in kinetics strongly suggests that the separation of TMD1, not S1 domains, is coupled to ion permeation. If the separation of S1 is also required, it must be coupled to TMD1 separation, and thus a stable CE state would be impossible. Third, if the S1 helix was truly important for channel gating, one would expect to have isolated numerous GOF- and LOF-effecting mutated Eco-*mscL* genes within the first 11 amino acids of the protein, where the significant interactions of the S1 helices were predicted to occur. However, in the original random mutagenesis study (Ou *et al.*, 1998), none of the 19 mutations isolated that effected a GOF phenotype were in this region. In a subsequent random mutagenesis study in which a rapid screening was used to isolate both GOF- and LOF-effecting mutants (Maurer and Dougherty, 2003), none of the 52 phenotype-effecting mutations were in this region; instead, the 26 mutations that were isolated in this domain gave no phenotype.

Hence, while the proposal of the CE state and S1 domains as a second gate is attractive given the importance of this region and the disulfide-trapping evidence that supports it, several other lines of evidence are more consistent with the proposal that this region serves a more structural role for the channel, such as being critical for correct folding or assembly of the complex. Given this apparent discrepancy, a more rigorous and detailed study of this region and its role in channel formation and/or function is needed.

4. Does the C-Terminal α-Helical Bundle Open?

In the original gating model for the Eco-MscL, the C-terminal bundle, now called S3, was predicted to separate on channel opening (Sukharev *et al.*, 2001b) (also see Fig. 2). However, the authors noted the sequence of the S3 of MscL was similar to that of the oligomerization domain of the cartilage protein COMP, which has a fivefold coiled-coil structure stabilized by apolar interactions inside the bundle and by salt bridges on the periphery

(Efimov *et al.*, 1996; Malashkevich *et al.*, 1996). The authors, therefore, tested if the S3 domain would associate in a similar structure, and if this structure is stable during channel gating (Anishkin *et al.*, 2003). Computer modeling suggested that this MscL structure could be quite stable, and disulfide trapping-experiments supported the predicted structure, and seemed to suggest that it remained so on gating. Using disulfide-trapping and biochemical analysis, the authors demonstrated that the predicted interactions between the S3 helices occurred quite efficiently under ambient conditions, strongly suggesting that their assumptions of the helical bundle are correct. Because of technical difficulties using oxidizing reagents in patch clamp, the authors made the assumption that "ambient" oxidative conditions for Eco-MscL channels undergoing biochemical analysis (which includes French press cellular disruption, solubilization, purification and reconstitution) would be the same for channels in spheroplasts subjected to patch-clamp buffer. The results were largely negative or subtle, but all were consistent with a stable S3 bundle during the gating process.

The proposed function of a stabilized S3 bundle is as a filtration device (Anishkin *et al.*, 2003). However, an early molecular sieving study suggested that the constriction site of the pore must be at least 30–40 Å (Cruickshank *et al.*, 1997)! In addition, studies comparing *mscL*-null strains with their parental have suggested that moderately sized proteins including thioredoxin, elongation factor Tu, and DnaK pass through the MscL channel on osmotic downshock (Ajouz *et al.*, 1998; Berrier *et al.*, 2000). At the time the study on the S3 bundle emerged, the ability of MscL to pass such large proteins had been put into question by another study that did not reproduce the results (Vazquez-Laslop *et al.*, 2001); however, it has been demonstrated that the discrepancy between the two studies is simply due to subtle differences in experimental approach (Ewis and Lu, 2005); these proteins are truly transported through the MscL channel on osmotic downshock. Hence, future studies will have to address the question: if the S3 bundle truly is stable on gating, how can such large molecules pass through the MscL channel?

5. Role for the Periplasmic Loop

Several studies have implied a function for the periplasmic loop located between TMD1 and TMD2. First, early mutagenesis demonstrated that substitutions in this region could influence the kinetics (Blount *et al.*, 1996c) and sensitivity (Ou *et al.*, 1998; Maurer *et al.*, 2000; Tsai *et al.*, 2005) of the channel. An additional study showed that when the channel is subjected to proteases, the channel sensitivity to membrane tension dramatically increases (Ajouz *et al.*, 2000). Consistent with this latter finding, as described in Section IV.A.1, when the Eco-MscL was divided in half so TMD1 and TMD2 were expressed independently (Park *et al.*, 2004), the channels that

were formed that had a similar conductance to the wild-type channel but had an increased sensitivity to membrane tension. Together, these data are consistent with the periplasmic loop playing the role of a torsional spring, inhibiting the channel from gating.

6. Asymmetric Movements

For simplicity, and given the radial symmetry of the channel, the model for gating the MscL channel assumes a smooth and coordinated movement of all of the subunits simultaneously; radial symmetry is largely maintained in the predicted transition states (with the exception of the S1 domains, which sequentially separate, as discussed in Section IV.A.2) (Sukharev *et al.*, 2001b). However, recent data has challenged the assumption that much of the channel remains radially symmetrical as the channel opens.

Few functional studies have been performed with the Tb-MscL channel because of its insensitivity to membrane tension (Section III.C.1), which leads to technical difficulties detecting channel activity prior to rupture of the patch (Moe *et al.*, 2000). However, a GOF-effecting mutated Tb-MscL channel, V15C, displayed the interesting property of efficient disulfide bridge formation as assayed by SDS-PAGE. Because this mutated channel contains only a single cysteine, these interactions presumably occurred between subunits within the complex. The resulting activity could easily be studied in patch clamp because the V15C-mutated channel showed a dramatic increase in sensitivity to membrane tension (Shapovalov *et al.*, 2003). The authors found that the channel often times did not go through a normal closure, but instead locked into an open state in response to pressure; the channel then slowly and irreversibly stabilized into partially open "signature events"; such events occurred even after the cessation of stimuli. The signature events become smaller with time, and subsequent opening of the channel is impossible. This phenomenon is not observed in the presence of reducing reagents. These data suggest that once a disulfide bridge is formed, the channel, on trying to close, locks into an unstable open structure, and presumably attains unnatural and irreversible conformational changes under the strain. Because the Tb-MscL crystal structure does not predict that the V15 residues can easily interact with one another within the channel complex, the findings suggest that asymmetric movements, in which one V15 approaches its neighbor, often occur on gating.

A similar and more recent study has been performed using the Eco-MscL (Iscla *et al.*, 2006). The analogous residue to the Tb-MscL V15 residue in the Eco-MscL channel, V17, did not demonstrate the same properties when mutated to cysteine, presumably because of a species difference. The authors, however, had scanned the area with cysteines and found a nearby mutation, N15C, which had interesting properties. Here, disulfide

trapping led to a channel that was more sensitive to membrane tension. The efficiency and rapid kinetics of this reaction suggested that the channel is trapped into a normal transition state that retains the capacity to attain both closed and fully open states. Furthermore, it is reversible by adding reducing agents. Similar to the Tb-MscL V15 residue, the position of the Eco-MscL N15 residue in structural models did not predict that this residue would interact unless asymmetric movements normally occur in the closed to open transition.

These data predict that although the MscL channel appears to have radial symmetry, it appears that gating is initiated with the movement of one region of one subunit of the protein. This region is just cytoplasmic to TMD1 and presumably is involved in or initiates the cascade of movements that leads to full channel opening.

B. Molecular Dynamic Simulations

Molecular dynamic simulation (MDS) is an approach in which detailed atomic interactions and structural mobility are assessed under different conditions by computer modeling. This is a relatively new approach that can help support or direct experimental studies. One of the limitations of this technique is that, because of the computer-processing requirements, only short stretches of time, normally a few tens of nanoseconds at best, can be followed, so often extreme or nonphysiological stimuli must be applied. The approach may vary by the presence or absence of solvent molecules or membrane, as well as the form and extent of stimulation to gate the channel. Several aspects of the structural changes that occur on MscL channel gating have been studied. For example, using either the Tb-MscL (Elmore and Dougherty, 2001; Gullingsrud *et al.*, 2001; Colombo *et al.*, 2003; Valadie *et al.*, 2003) or Eco-MscL (Kong *et al.*, 2002; Gullingsrud and Schulten, 2003; Valadie *et al.*, 2003) protein structural models (Figs. 1 and 2), researchers have obtained data that support the hypothesis that the transmembrane helices tilt within the lipid bilayer on gating and that the lumen of the open pore is generated primarily by TMD1, as discussed in Section IV.A.1. In addition, one MDS study also showed the clockwise rotation of the TMD1 and exposure of the I24 residue (Colombo *et al.*, 2003), as discussed in Section IV.A.2. In another experiment (Elmore and Dougherty, 2001), the C-terminal region of the protein was assayed under different pH conditions and it was found that the bundle was stable only at the low pH in which the Tb-MscL was crystallized; these data would be consistent with an alternative structure for this region, as has been proposed (Anishkin *et al.*, 2003) and is discussed in Section IV.A.4. There are, however, inconsistencies between

studies for the presence or absence of the stabilized CE structure and putative S1 helices forming a second gate of the Eco-MscL channel, as discussed in Section IV.A.3. For example, starting from the proposed Eco-MscL-closed structure (Fig. 2), one study found a total expansion of the pore could occur without seeing a disruption of the S1 bundle (Gullingsrud and Schulten, 2003), while another group found that the S1 and TM1 do not behave as two independent gates, but the separation of these domains is coupled (Kong *et al.*, 2002). In one experiment, the membrane was curved to stimulate the channel; this study found significant movements within the periplasmic loop structure, which would be consistent for the "torsional spring" function discussed in Section IV.A.5 (Meyer *et al.*, 2006). Many of the studies found asymmetric movements of the complex, as opposed to maintaining radial symmetry, as the channel opens (Bilston and Mylvaganam, 2002; Kong *et al.*, 2002; Colombo *et al.*, 2003), thus supporting the possibility of the asymmetric movements discussed in Section IV.A.

V. PHYSICAL CUES FOR MscL CHANNEL GATING: PROTEIN–LIPID INTERACTIONS

Perhaps one of the more intriguing questions concerning mechanosensors is: what exactly are they sensing? Clues have been derived from several studies using a variety of approaches. The data thus far suggest that the MscL channel is able to directly sense biophysical changes in its membrane environment.

A. Studies of the Energetic and Spatial Parameters for MscL Gating

When the MscL channel is stimulated in a membrane, the amount of stimulus, in pressure, can be measured by a pressure transducer. In addition, with the proper equipment, the patched membrane can be imaged and the radius of curvature also measured. Using these parameters, tension within the membrane can be calculated using Laplace's law: tension in the membrane equals the pressure across it times the radius of curvature divided by 2. A Boltzmann model for the relationship of the probability of the channel opening vs the membrane tension can then be used to plot and study the energetic and spatial parameters for the gating of the Eco-MscL channel. This analysis was performed (Sukharev *et al.*, 1999b), updated (Chiang *et al.*, 2004), and has been used for some of the GOF-mutated channels (Anishkin *et al.*, 2005). From these analyses, the current-derived values for opening the wild-type channel hold that it takes ~7–13 dynes/cm^2 of tension in the

membrane to achieve a 50% probability of channel gating, the energy required to gate the channel at this level (ΔE) is $51 \pm 13kT$, and the change in area (ΔA) is 20 ± 5 nm^2. Note that the latter parameter is consistent with current models for the approximate pore size of the open channel. In addition, when the rate constants for achieving specific substates of the channel are plotted against membrane tension, it appears that it is only the closed to first substate conversion that is responsive to tension; once the channel has begun ion permeation, the progression to the different substates occurs independent of external stimulus.

B. *Does MscL Sense the Pressure Across the Membrane or the Tension Within It?*

As mentioned in Section V.A, pressure across the membrane and tension within it are related, but not the same. According to Laplace's law, tension in the membrane equals the pressure across it times the radius of curvature divided by 2. The first study to determine if an MS channel was sensing pressure across the membrane or tension within it was performed on a yeast channel (Gustin *et al.*, 1988). The authors performed the whole-cell patch technique with positive pressure in the patch to observe the channels. Three cells of very different diameter, which would thus have a different radius of curvature, were assayed. When the probability of opening, P_o, was plotted against the pressure, three distinct curves were found. However, when the P_o was plotted against the calculated tension, the data collapsed to form a single curve. Thus, this yeast channel appears to sense membrane tension.

When the spatial and energetic parameters were measured for Eco-MscL (Section V.A), the data were consistent with MscL sensing membrane tension and the authors assumed this was the case, but it was not formally demonstrated (Sukharev *et al.*, 1999b; Chiang *et al.*, 2004). However, using appropriate imaging equipment so that the curvature of the patch can be measured, an experiment similar to that performed in the yeast system was performed, where patches with quite different radius of curvature were compared (Moe and Blount, 2005). These experiments demonstrated that the MscL channel does indeed sense tension within the membrane, not the pressure across it.

C. *Sensing the Biophysical Properties of the Membrane*

Soon after the identification of MS channel activities in bacterial membranes, it was realized that changes in the biophysical properties of the membrane are important for channel stimulation. Amphipaths, having both

hydrophilic and hydrophobic character, will intercalate into membranes and thus change the lateral pressure profile within the membrane. A combination of a charge on the amphipath and the potential across the membrane can lead to an amphipath preferentially partitioning into one leaflet or the other. An early study determined the effect of addition of amphipaths on bacterial MS channels (Martinac *et al.*, 1990). This study found that addition of either positively charged, negatively charged, or uncharged amphipaths could increase the sensitivity of the channel to stimuli. After the activation of the channel by an amphipath of one charge, its replacement with an amphipath of the opposite charge would partially reverse the effects prior to activation, presumably because the amphipaths would partition within opposing sides of the membrane, thus canceling the effect. While this original study was performed prior to the distinction between the MscS and MscL channel activity, and thus was probably studying primarily MscS, a similar study has been performed for the purified Eco-MscL where channel activity and structure were monitored by patch clamp and EPR, respectively (Perozo *et al.*, 2002b). The findings were consistent, demonstrating that lysophospholipids, which are strongly amphipathic molecules, could gate the Eco-MscL channel. The same study to determine the influence of the possible thinning of the membrane, or hydrophobic mismatch, on channel gating also reconstituted the Eco-MscL channel into lipids with varying chain length. While the sensitivity of the channel increased with decreasing chain length, the channel did not spontaneously gate. Hence, it appears that while hydrophobic mismatch may play some role in channel sensitivity, it appears that the true stimuli for the channel are changes in the biophysical properties of the membrane and perhaps modification of the lateral pressure profile.

D. Specific Protein–Lipid Interactions

Given that the MscL channel detects tension in the membrane, is modified by hydrophobic mismatch, and appears to detect biophysical changes in the membrane, it is tempting to speculate that there are specific protein–lipid interactions involved in the ability of the channel to sense stimuli. Several studies have suggested this could be the case. For example, it was noted in one of the random mutagenesis studies that LOF-effecting mutations often occurred near the proposed site of interaction with membrane lipid headgroups, suggesting such specific interactions may occur (Maurer and Dougherty, 2003). In another study looking for intragenic suppressors for a GOF mutant, I41N, the authors noted that some of the suppressing mutations were clustered on the periplasmic side of the transmembrane domains, again near the predicted headgroups (Yoshimura *et al.*, 2004). The authors, therefore,

performed an asparagine scan of the region and found that mutation of many of the residues predicted to face the lipid led to channels that were less functional (Fig. 5). While the study is well done and the interpretation attractive, some predictions have not yet been fulfilled by other studies, as one might have expected. For instance, a similar clustering was not noted in another intragenic suppressors study (Li *et al.*, 2004); but this may simply be because in the latter study partial suppressors were selected, and neither study saturated their screen. In addition, a similar scanning of the region with cysteines did not find the same results (Levin and Blount, 2004). In another set of studies, tryptophan mutagenesis and tryptophan fluorescence spectroscopy revealed a relatively nonspecific association between the protein and uncharged lipids, but a highly specific binding with anionic lipids. Three positively charged residues in a cluster near the cytoplasmic end of TMD2

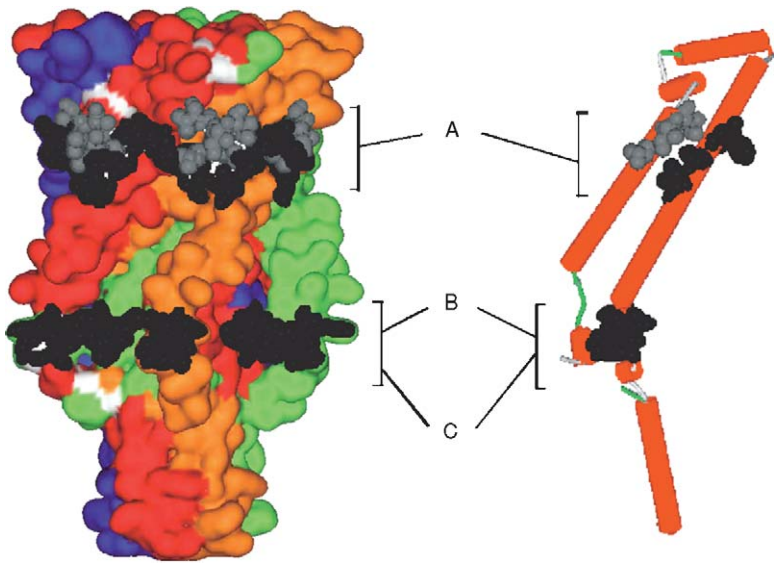


FIGURE 5 Protein–lipid interactions in the MscL channel. Residues implicated in lipid binding are in a side view of the channel (left) with individual subunits indicated by color and, for clarity, on a single subunit (right). Mutation of residues lining the periplasmic rim of the channel (A) yielded an LOF phenotype, presumably through disruption of lipid binding (Yoshimura *et al.*, 2004). These residues form the periplasmic end of TMD1 (gray) and TMD2 (black). Tryptophan fluorescence spectroscopy revealed heterogeneity in lipid binding to the channel (Powl *et al.*, 2005). No binding preference was detected for uncharged lipids, however, anionic lipids were found to associate strongly with a pocket of charged residues at the cytoplasmic end of TMD2 (B). This charged pocket lies at the beginning of a charge cluster, RKKEE, postulated to form a pH-sensing domain that regulates the channel sensitivity (C) (Kloda *et al.*, 2006).

were implicated (Powl *et al.*, 2005) (Fig. 5). Disruption of one charge in this cluster significantly reduced this anionic lipid binding. Further manipulation of this pocket was found to perturb lipid association and yield a GOF phenotype, possibly reflecting a conformational disruption of the protein. Interestingly, this charge cluster is proximal to, and included in, a charged domain that has been postulated to function as a pH sensor. In this latter study, this and other charges just cytoplasmic to TMD2 were shown to lead to a pH modification of channel sensitivity in patch clamp. The authors suggested that this region may interact with charted lipids in a pH-sensitive manner (Kloda *et al.*, 2006). A previous study was consistent with the findings shown for the wild-type channel (Iscla *et al.*, 2004); however, a functional role for this potential pH modulation has yet to be demonstrated *in vivo*.

Together the data discussed above are beginning to give a glimpse of possible interactions between the MscL protein and the headgroups of the lipids. If the interactions are specific, as some of the studies imply, then one would anticipate that changing lipid headgroups would have influences at least as profound as mutation of the protein. To test this hypothesis, one group assessed the influence of changing lipid composition on channel function (Moe and Blount, 2005). Lipids with phosphatidylcholine headgroups are not generated by bacteria and were, therefore, used as a standard. The addition of phosphatidylserine, which contains a negative charge, did not influence the tension needed to gate the channel. The two major lipid headgroups in the *E. coli* cytoplasmic membrane were also tested. The major negatively charged *E. coli* lipid headgroup, phosphatidylglycerol also had no effect. The other major headgroup, phosphatidylethanolamine, actually led to a channel activity with at a *lower* rather than higher sensitivity to membrane tension, suggesting that this lipid effects altered activity through changes in the biophysical properties of the membrane rather than through MscL-lipid specific interactions. This study cannot rule out the possibilities that there are nonfunctional interactions or that some minor lipid headgroup plays a positive role in MscL channel function. However, it does appear that none of the major *E. coli* lipid headgroups specifically interact functionally with residues of the MscL channel.

VI. MscL AS A POSSIBLE NANOSENSOR

As nanotechnology increases in promise and scope, researchers are beginning to realize that biosensors may fill the role of nanosensors in many systems, allowing electrical current, or the release of small chemicals or drugs on stimulation. MscL, with its large pore size and streamlined structure, is a prime candidate for such a nanosensor.

In several experiments, the modality of the channel has been measured or changed. One study, using whole cells from wild-type and an MscL-null mutant, implied that the MscL channel responds to increases in temperature (Jones *et al.*, 2000); however, recent experiments have found the opposite influence of temperature on reconstituted channels (Li *et al.*, unpublished results), suggesting the cellular responses may be indirect and not an inherent property of the simplest lipid-protein system. In other studies, either the lipid composition (Folgering *et al.*, 2004) or protein structure (Koçer *et al.*, 2005) has been modified to generate a system that is sensitive to light of specific wavelengths. Finally, mutagenesis (Iscla *et al.*, 2004) and protein modification (Koçer *et al.*, 2006) have made channels with an increased sensitivity or spontaneous activity at a specific pH. The channel has been shown to function while reconstituted in lipid vesicles (Koçer *et al.*, 2005, 2006); unfortunately, the one published attempt at reconstituting the channel into a nonlipid matrix led to channels that were frozen in specific conformations, depending on the pressures used to generate the matrix (Ornatska *et al.*, 2003).

Researchers have also investigated whether the MscL channel can be synthesized *in vitro* rather than producing it in a biological system; the obvious advantage would be that large quantities could be generated. One study demonstrated that the channel can be synthesized in a cell-free system in the presence of detergent rather than phospholipid membranes; a functional channel is produced as assayed by patch clamp after reconstituted into membranes (Berrier *et al.*, 2004). In other studies, the channel was generated by total chemical synthesis (Clayton *et al.*, 2004); in a later work, such a synthesized channel was shown to be able to form functional channels when reconstituted into lipid membranes (Becker *et al.*, 2004).

VII. CONCLUSIONS

MscL has given us our first and deepest glimpse into the workings of a mechanosensory channel. In less than 13 years, we have progressed from identifying the gene that encodes the MscL activity to detailed structural and mechanistic models. However, there are still many details to be worked out and questions to be addressed. It remains the challenge of the future to determine exactly how the channel detects membrane tension and the precise structural rearrangements the channel undergoes on stimulation.

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