Sensing bilayer tension: bacterial mechanosensitive channels and their gating mechanisms

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Abstract
Mechanosensitive channels sense and respond to changes in bilayer tension. In many respects, this is a unique property: the changes in membrane tension gate the channel, leading to the transient formation of open non-selective pores. Pore diameter is also high for the bacterial channels studied, MscS and MscL. Consequently, in cells, gating has severe consequences for energetics and homeostasis, since membrane depolarisation and modification of cytoplasmic ionic composition is an immediate consequence. Protection against disruption of cellular integrity, which is the function of the major channels, provides a strong evolutionary rationale for possession of such disruptive channels. The elegant crystal structures for these channels has opened the way to detailed investigations that combine molecular genetics with electrophysiology and studies of cellular behaviour. In the present article, the focus is primarily on the structure of MscS, the small mechanosensitive channel. The description of the structure is accompanied by discussion of the major sites of channel-lipid interaction and reasoned, but limited, speculation on the potential mechanisms of tension sensing leading to gating.

Introduction
Mechanosensitive channels sense changes in the tension in the membrane bilayer [1]. In higher organisms the channels have diverse functions, but in bacteria, they are required for the maintenance of cell integrity during severe shifts in osmolarity, specifically during rapid transitions from high to low osmolarity (hyper-osmotic shock) [2]. Channel gating leads to the transient formation of high-conductance pores in the cytoplasmic membrane on a timescale of a few microseconds [3]. Detailed analyses of the permeability properties of all of the bacterial channels identified have yet to be completed. However, pores are large, probably ranging from 8 to 30 Å (1 Å = 0.1 nm) in diameter [4]. Consequently, the pores are predominantly non-selective, except on the basis of the size of the ion or organic solute that they pass. The best-studied channels are those encoded by the mscL gene (MscL) and the yggB gene (MscS) [5]. Crystal structures for the Mycobacterium MscL channel and the Escherichia coli MscS channel have been solved to ∼3.5 Å and reveal the channels to be a homopentamer (MscL) and a homoheptamer (MscS) [6,7]. Most organisms possess both MscL and MscS, but there are often several additional MscS homologues, which form a large family of structurally related proteins [8]. Larger members of this family (>300 residues) bear a domain equivalent to MscS at their C-terminal end, but the sequences at the N-terminus are quite diverse [2]. Two of these MscS homologues from E. coli, YbdG and MscK, have been studied [9,10] and have been shown to be active, and, for both homologues, mutations in the ‘MscS domain’ modify the gating of the channel consistent with this proposed role (M.D. Edwards, U. Schumann, S. Black, S. Miller and I.R. Booth, unpublished work). Different gene products form channels with different sensitivities to tension and each with a unique pore size. There is no evidence for the formation of hetero-oligomers between different gene products.

Most studies on mechanosensitive channels have used proteins from E. coli applying a range of techniques, but additional insights into MscL have been derived from investigations of the Mycobacterium and Staphylococcus channels [6,11–14]. Critical residues within the E. coli protein have been identified from mutagenic studies, and clues to the molecular motions have been derived from ESR using probes attached to cysteine residues introduced by site-directed mutagenesis [15–21]. Paradoxically, given the importance of tension sensing, considerably fewer studies have tried to understand how the proteins interact with the lipid bilayer [13,22–24]. In the present article, we principally seek to describe our understanding of the MscS channel. It is clear that MscL should share some fundamental similarities with regard to lipid–protein interactions, but it is structurally and mechanistically different and it is hazardous to extrapolate too much from MscL to MscS. Moreover, MscS is a large and complex family of proteins exhibiting considerable diversity of structure, where insights into the differences in mechanisms between large and small homologues remain to be discovered. Work on MscL is described elsewhere in this...
The structure of MscS

Recognition that the *yggB* gene of *E. coli* encodes the MscS channel monomer paved the way to both the structure and to underpinning biochemical experiments to understand the structural changes during gating [2,7]. Rees and colleagues solved the structure for the *E. coli* MscS channel to ∼3.5 Å [7]. MscS is a homoheptameric transmembrane protein with a large cytoplasmic domain. The membrane domain is composed of three α-helices per subunit, and the cytoplasmic domain is predominantly composed of β-sheets (Figure 1). Overall, however, each subunit (286 amino acids in *E. coli*) exhibits a complex domain structure. Both the N-terminal sequence (residues 1–26) and the last six residues of the C-terminal domain were not modelled in the crystal structure because of the lack of well-defined density corresponding to these sequences. However, models have been generated for the N-terminal region using EPR and molecular dynamics, and function is implied from the properties of mutants in this region [25].

Membrane domain

Each monomer contributes three helices (TM1–TM3) to the membrane domain. Density for TM1–TM2 (residues 27–91) was less well defined than for the main body of the protein [7,26]. They form a sensor that interacts with the lipids of the bilayer (see below) [7]. During gating, the TM1–TM2 domain appears to move as a rigid body that transmits tension to the pore via a short sequence (residues 92–95) [26]. In the original crystal structure, this region exists in extended chain conformation. Mutations in this sequence cause the channel to gate at lower bilayer tension [27,28]. The third helix, TM3, has two sections: residues 96–112 (TM3a) and 114–127 (TM3b). TM3a helices line the pore and are aligned at 25–35° to the membrane normal, which is also the pore axis. The residues lining the pore are hydrophobic. Two rings of leucine residues (Leu105 and Leu109) form a pore constriction that must be expanded to generate the open pore. The packing surface between the adjacent TM3a helices is marked by a conserved alanine/glycine pattern that is a critical determinant of the gating tension [7,29]. TM3a ends with an Asn-Gly sequence that forms a bend leading to TM3b, which lies along the surface of the bilayer (see below).

Initially, there were some doubts regarding the nature of this form of MscS, i.e. whether it depicted the open or closed form of the channel [7,30]. A range of *in silico* analyses of the diameter of the pore indicated that the pore was very narrow and was too small to account for the conductance of the channel [30–32]. Additionally, the whole pore had been noted to be extremely hydrophobic, and molecular dynamics simulations led to the suggestion that the pore was closed by the inability to sustain a water column, the so-called ‘vapour lock’ [30,33]. A subsequent structure (3.45 Å resolution), derived from the crystallization of an MscS A106V mutant, trapped MscS in

![Figure 1](image-url)
an conducting, open, state [26]. Here a pore approximately 13 Å in diameter was estimated using the HOLE program, compared with minimum 4.8 Å diameter estimated from the original structure of the wild-type protein [34,35]. The expanded pore diameter of the A106V structure is in the right range for the measured values of the conductance, and the expansion of the pore is of the magnitude that had previously been predicted for the closed-to-open transition [36].

**TM3b**

The role of TM3b in MscS is controversial and our understanding of the roles played by this sequence is still evolving [37–39]. Genetic analysis has led to considerable speculation regarding the role of the Asn-Gly hinge and TM3b, which is dealt with below. Asn,

112, the final residue of TM3a, generates a helix break, which is essential for channel function, and creates a sharp bend around residue 113 [7]. Asn, 112 is essential for channel function, whereas amino acid substitutions at Gly, 113 are highly tolerated [37,40]. In both wild-type and A106V structures, a significant cavity exists where any side chain extending from position 113 would be placed. Indeed, a G113W mutant, which represents the largest amino acid substitution possible, forms a stable functional heptameric channel protein (M.D. Edwards, T. Rasmussen, S. Miller and I.R. Booth, unpublished work). The C-terminal end of TM3b has a pair of highly conserved basic residues (Arg and Arg). Mutation of either Arg or Arg to aspartate causes a slight loss of function in the channel [41]. These residues may have important roles in gating transitions through interactions with lipid headgroups.

**The cytoplasmic domain (vestibule)**

The vestibule structure is formed by the two predominantly β-sheet domains (Figure 1). The upper ‘middle β-domain’ is wholly β-sheet, and the seven subunits produce a relatively rigid cylindrical β-domain that surrounds the axis of the pore. It is connected to the lower ‘αβ-domain’ by a short linker. The edges of the two domains, in combination with the connecting linker, define the perimeter of the lateral portals that connect the transmembrane pore to the cytoplasm [7]. During activation of the channels in the cell, considerable ion flux must take place through these portals to account for the rapid depletion of the cytoplasmic ion pools [34]. The vestibule inner surface is predominantly polar in character, but with few charged residues; consequently, the surface will not exhibit strong ion-binding properties. The lower domain is potentially more flexible than the upper domain due to the attachments to the ‘middle β-domain’ and the terminal β-barrel via extended peptide linkers [28]. Previously, we have referred to this as a Chinese lantern arrangement, indicating the potential for the vertical movement (in the direction of the membrane surface) that could lead to narrowing of the portals [42]. Various findings are consistent with the structure of this domain affecting the gating threshold for the channel, but the indirect nature of the assays makes explicit interpretation difficult.

**The closed-to-open transition**

The availability of two structures allows some prediction of the structural transition associated with gating. Thus, in the A106V structure the TM1–TM2 helices have rotated as a rigid body [26]. Although crystal structures are static representations of dynamic systems, comparison of the two available MscS structures does suggest significance of the TM1–TM2 sensor consistent with its ability to sense and respond to changes in its lipid context [7,26]. The movement of TM1–TM2 would result in a conformation of the linker to TM3a. What is clear from the more recent structure is that TM3a helices can tilt and separate [26]. In the closed structure, these helices achieve the very tight packing due to the match between surfaces populated with alanine (knobs) and glycine (grooves) residues [7,43]. In the A106V structure, the helices have separated, rotated and moved radially outwards and, in addition, they are aligned with the membrane normal [26]. It is this series of positional changes that generates the open pore by increasing the separation of the hydrophobic rings generated by Leu and Leu. The spatial separation of the TM3a helices is sustained by interactions at the base of TM3a between Ala and with Leu of TM3b of the adjacent subunit. At the upper end, there are two groups of interactions that could stabilize the open state: Ile with Ala/Ser of the next subunit and Thr with Gln of the adjacent subunit. The importance of these last interactions has not been tested in detail.

In conclusion, the structure of MscS provides a stable framework for creating a large pore in the cytoplasmic membrane in response to changes in bilayer tension. Rapid electrophysiological measurements have indicated that the transition from the closed to the open state can take place in a few microseconds [3]. Measurements made with the channel under constant moderate tension indicate that the channels open and close stochastically and have a potentially long open dwell time (>100 ms) [29]. At higher values of tension, alternative closed states may be attained, some of which are prevented from rapidly re-attaining the open state [38,44–46]. However, these observations must be tempered by the fact that, in cells, the open channel rapidly dissipates the transmembrane turgor pressure that is the physiological signal for channel activation. Thus the physiological significance of inactivated states in vivo is uncertain.

**Lipid–protein interactions in MscS**

Mechanosensitive channels gate in three specific circumstances. In cells, the channels gate when transmembrane turgor rises rapidly. This can be triggered by adapting cells to high osmolarity followed by rapid transfer into medium of low osmolarity [47,48]. Turgor increases due to the rapid inflow of water down the osmotic gradient generated by the high intracellular concentrations of osmotically active solutes accumulated by growing cells. The turgor pressure rises rapidly and has the potential to exceed 30 atm...
The gating of the channels allows the rapid release of the accumulated solutes, which reduces the driving force for water entry and therefore lowers turgor pressure. Clearly, the effects of turgor must be converted into variation of lipid bilayer tension such that the channels gate. In membrane patches, the channels can be triggered to gate either by exerting pressure on to the patch or by the insertion of amphipaths, such as lysophosphatidylcholine, into the lipid bilayer [50,51]. Pressure is exerted on to the patch by creation of negative pressure in the pipette to which the patch is attached, leading to an outwardly directed pressure equivalent to turgor in cells. Amphipaths insert asymmetrically into the patch either by their inclusion in the pipette buffer (exposure to the outer leaflet of the membrane bilayer) or by inclusion in the bath. Their effects take some time to be seen, but generally result in lower pressure being required to gate the channels. In all three scenarios, the effects of pressure on the membrane must modify the lipid organization in proximity to the channel and the channel must possess a mechanism to sense the changes.

MscS has three principal lipid–protein interfaces. The TM1–TM2 helix pair was originally proposed to sense membrane tension [7]. It was noted from the crystal structure that TM1–TM2 acted as an independent domain and that its movements in the lipid bilayer would be transmitted to the pore helices through the linker between TM2 and TM3a. This may be the principal sensory domain of the MscS channel. The amino acid composition of TM1 and TM2 exhibits an extreme asymmetry. Low polarity is observed in the periplasmic half of both helices, and highly polar residues are found in the cytoplasmic half (Figure 2). This should predispose TM1–TM2 to interact differentially with the lipid headgroups at the periplasmic and cytoplasmic surfaces, which seems likely to be an intrinsic element of the lipid tension sensing. As indicated above, TM3b is predicted to lie along the inner membrane leaflet and presents a hydrophobic surface to the bilayer. The distal end (with respect to the pore) of TM3b has a pair of arginine residues that could interact with lipid headgroups and provide an anchor (Figure 3A) (see below). Finally, residues 1–26 were not modelled in the crystal structure because of a lack of density corresponding to this sequence [7]. Although this implies a degree of motility in this region, structured models have been predicted [25]. Critically, mutations that replace Trp16 with other residues have been shown to modify MscS gating in response to pressure [52]. Tryptophan residues are known to have a propensity to burrow into the lipid bilayer and to interact with the phospholipid headgroups.

Substitution of tyrosine for tryptophan, which can also interact with lipid headgroups, increases the pressure required to gate the channel. Mutations that retain the hydrophobic character of tryptophan, but lack the ability to interact with lipid headgroups (phenylalanine or leucine) have more severe gating defects than the tryptophan–to-tyrosine change, consistent with lipid headgroup interactions at this position being critical to MscS function [52].

Genetic studies have explicitly address the interactions of TM1–TM2 with lipids [41,53]. Yoshimura and colleagues performed asparagine substitution mutagenesis in which the residues of TM1–TM2 were systematically replaced by asparagine [53]. The asparagine side chain has the capacity to hydrogen-bond to lipid headgroups and also with backbone carbonyls and amides of TM3a [54–56]. Yoshimura and colleagues noted position-specific changes in the gating pressure of the modified channels (Figure 3B) [53]. Asparagine positioned at the periphery of TM1 or TM2 causes the channel to become less sensitive to membrane tension. Introduction of two or more mutations at the equivalent positions relative to the bilayer causes complete loss of sensitivity to pressure. Thus F68N combined with A51N, where both mutations affect residues at the cytoplasmic end of the TM1–TM2 helix pair, was very insensitive to pressure and exhibited a greater change in pressure-sensitivity than either single mutant alone. In addition, when two mutations were introduced, but at opposite ends of the TM1–TM2 helix pair (e.g. F68N/I27N), there was no significant additive effect of the mutations on pressure sensitivity. An opposite effect was observed when the mutations were introduced towards the middle of the TM1–TM2 helix pair. Now the single mutations (e.g. I39N) caused the channel to gate at lower pressure. These data are supported by other studies in which mutations that increased gating were isolated [57]. Yoshimura and colleagues also confirmed that asparagine could be replaced by other charged residues (aspartate and lysine), but that the phenotype was not significantly altered by mutations that led to substitution of one hydrophobic residue for another [53]. Similar studies on Mscl also identified that introduction of asparagine residues close to the periplasmic rim of TM1 and TM2 inactivated the channels by changing the tension sensitivity [58].

The original interpretation of these studies was that the hydrophobicity of the residue was important, but the studies

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**Figure 2 | Surface representation of MscS membrane domain**
Residues up to 113 are shown on the basis of the closed wild-type structure of *E. coli* MscS. Oxygen atoms are red, nitrogen atoms are blue, sulfur atoms are yellow, and carbon atoms are grey. The Figure was generated with PyMOL (http://www.pymol.org).
Figure 3 | Lipid contact zones for the MscS channel

(A) Structure of TM3b. The structure is depicted in ribbon format, and the location of the hydrophobic amino acids side chains that are predicted to interact with the lipid bilayer are shown in black. The two highly conserved arginine residues (Arg^{128} and Arg^{131}) are shown in red. The orientation of these residues in the crystal structure may be explained by the presence of detergent (and/or lipid) in the crystals, but they could adopt the same orientation to interact with the lipid bilayer.

(B) Positions of some of the asparagine-substitution mutants created by Yoshimura and colleagues in MscS [53]. Residues in red are strongly inhibitory, whereas those in blue activate MscS (gain-of-function). Note that in the membrane, TM1–TM2 may be turned slightly to pack against the outer surface of the TM3a helix. This has the effect of burying the ‘red’ residues against TM3a. In contrast, the blue residues always face the lipid bilayer whichever packing of TM1–TM2 with TM3a is preferred by the experimentalist.

did not provide a mechanistic interpretation of the mutations. The elucidation of the structures of many inner and outer membrane proteins has led to a general ‘rule’ about the location of tryptophan and tyrosine residues (and, to a lesser extent, asparagine and glutamine) [59,60]. In the majority of membrane proteins that have been studied, the tyrosine and tryptophan residues are found in positions proximal to the phospholipid headgroups. Both of the hydrophobic parts of the side chain tunnel down towards the fatty acid side chains of the lipids, whereas the polar groups form hydrogen bonds to oxygen atoms in the headgroups of the phospholipids. One of the most marked observations regarding both MscS and MscL was the extremely limited numbers of tryptophan or tyrosine residues in the lipid exposed regions of the proteins. MscS has a single tyrosine (Y75) and a single tryptophan (W16) residue per subunit in membrane regions. Mutations at either of these positions modify channel gating, consistent with an interaction with the phospholipid headgroups [52]. However, the striking feature of the structures of TM1–TM2 in MscS is the extreme asymmetry in the distribution of polar amino acid residues at the periphery of these helices (Figure 2). This creates the potential for these helices to move within the bilayer in response to changes in membrane tension, causing the necessary changes in the linker TM2–TM3A that is required to open the pore. Thus one would argue that a fundamental aspect of mechanosensation for MscS (and also MscL) is the lack of strong interactions between the periphery of the TM1–TM2 sensor paddle with the lipid headgroups. This could allow for significant asymmetry in the movement in the TM1–TM2 domain in response to changes in lipid packing consequent to high turgor. However, it is still not clear how lipid packing is sensed. The structure of MscS implies a periplasmic pivoting of TM1–TM2 with the cytoplasmic ends describing an arc, which at its most extreme may involve TM1–TM2 passing across TM3b, as described in the A106V structure [26]. Mutant studies support this model, with tension being passed through the stretched protein strand that connects TM1–TM2 to TM3a, leading to the rotation and separation of TM3a helices. The observation that the introduction of asparagine (or charged amino acids) [53,57] into the middle region of these helices leads to gating at low applied pressure (gain-of-function) is also consistent with this model. The requirement for hydrogen-bond formation (for the minimum energy state of the protein) between asparagine (or aspartate or lysine) is satisfied via interaction with the phospholipid headgroups, which requires a positional change in the TM1–TM2 equivalent to the pivot arising from changes in lipid packing. Blount and colleagues also identified that introduction of polar residues (V40D or V40K mutations) in the middle of TM1 caused MscS to gate at lower tensions, consistent with the formation of TM1–lipid headgroup interactions, leading to repositioning of TM1–TM2 towards the ‘open state’ [57]. We propose that this permanent change in orientation is equivalent to the first part of the molecular motion associated with gating of the wild-type channel, hence less energy is required from membrane tension to achieve the open state. The loss-of-function asparagine mutants could arise by anchoring TM1–TM2 to lipid headgroups due to the peripheral location of the inhibitory mutations. Alternatively,
these asparagine residues may form hydrogen bonds to the peptide backbone of TM3a, thereby increasing the energy needed to allow the pivoting of TM1–TM2 and gate the channel.

A second study investigated the roles of the highly conserved basic residues (Arg\textsuperscript{128} and Arg\textsuperscript{131}) at the C-terminal end of TM3b \[41\]. Mutation of either Arg\textsuperscript{128} or Arg\textsuperscript{131} to aspartate causes a slight loss of function in the channel. The phenotype of the arginine-to-aspartate mutants has been interpreted in terms of breaking a salt bridge interaction with acidic residues at the base of the TM1–TM2 sensor paddle (Asp\textsuperscript{62}) \[61\]. Such a role is problematic in that crystal structures suggest these residues to be at least 5 Å apart, which would provide only for a very weak interaction. Molecular dynamics simulations have suggested that TM1–TM2 is packed tightly against the TM3a pore when the protein is inserted in the lipid bilayer, which would only exacerbate the problems of making a salt bridge between Arg\textsuperscript{128}/Arg\textsuperscript{131} and Asp\textsuperscript{62} in TM1–TM2. These residues may have important roles in gating transitions through interactions with lipid headgroups. In the symmetrical homomultimer, these arginine residues form a ring of 14 positive charges that can interact with the phospholipid headgroups and thereby anchor the extremities of TM3b. During the formation of the open state, there is rotation of TM3a and this ‘turning force’ must be absorbed by TM3b to attain stability in the open state. The hydrophobic residues of TM3b are likely to be anchored in the bilayer lipid phase, whereas the interactions of Arg\textsuperscript{128} and Arg\textsuperscript{131} with lipid headgroups would provide further resistance to rotation of TM3b. Hence any tendency of TM3b to rotate would be resisted, leading to stabilization of the open state. Support for this analysis comes from insertion of helix-breaking residues into TM3b close to the pore axis \[37,46\]. These mutant channels almost invariably display instability of the open state consistent with loss of resistance within TM3b. Recent work that has deployed selection for increased gating at low tension to identify mutations from a random genetic pool supports the potential importance of these residues in stabilizing the closed state \[39\].

The larger members of the MscS family pose an intriguing aspect to the problem of protein–lipid interactions and tension sensing \[10\]. The majority of MscS homologues possess multiple additional N-terminal transmembrane helices. These proteins can be broadly divided into two classes: those that have an additional two helices per monomer and those that have eight extra helices per subunit (Figure 4). Surrounding the core MscS domain with a number of transmembrane helices generates two potential problems. First, even for the homologues with just two extra helices per subunit, there is the possibility of a ring of protein that isolates the TM1–TM2 sensor from the lipid bilayer. Secondly, it raises the possibility that, in the larger channels, the sensor is not the helices immediately adjacent to TM3a, but is more remote and requires a more complex mechanism for transmission of the tension to the pore. Studying these proteins offers considerable rewards for the understanding of the mechanism of tension sensing by mechanosensitive channels.

In conclusion, major progress has been made on many aspects of the mechanism of pore formation by mechanosensitive channels. However, the central conundrum of lipid–protein interactions and tension sensing has not yet been solved. It is an important problem, since this class of membrane protein has potentially the most dynamic set of interactions with the lipid bilayer. Their mechanism involves sensing changes in tension, which probably reflect changes in lipid packing or organization. Although membrane proteins do sometimes display changes in activity in the context of changing the lipid composition of the membrane, in particular there is selectivity for lipid headgroups, there is little evidence for this being a dynamic property of the membrane (although for a dissenting view, see \[62\]). This makes mechanosensitive channels almost unique among membrane proteins since the lipid changes from being a ‘passive’ context in which the protein functions, to an active component that by its own modulation determines protein activity.

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