CHAPTER 10

Structure–Function Relations of MscS

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I. OVERVIEW

The mechanosensitive (MS) channel MscS is the more widespread of two major MS channels that have been characterized. MscS-like proteins have been discovered in bacteria, archaea, yeasts and fungi, and in plants. In most organisms, multiple homologues have been found, although few have been characterized in detail. In *Escherichia coli*, where most work has been carried out, there are three small MscS homologues of 286 (MscS, YggB), 343 (YnaI), and 415 (YbdG) amino acids. In addition, there are three members

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of an MscS subfamily, typified by MscK (KefA) in *E. coli*, which are restricted to Gram-negative bacteria and which feature a more complex organization in the membrane. These proteins are between 741 (YbiO) and 1120 (MscK) residues in length and possess both a large periplasmic domain and additional membrane domains N-terminal to their "MscS channel" domain. Of the six *E. coli* proteins of the MscS family discovered to date, most is known about MscS and MscK. In this chapter, we will present current views on the function, expression, structure, and mechanism of the MscS proteins, making reference to MscK where appropriate.

II. INTRODUCTION

MS channels in bacterial cells fall into two major categories defined by their core structures: MscL and MscS (Chang *et al.*, 1998; Bass *et al.*, 2002). Both have now been studied extensively using molecular genetics allied to electrophysiology and protein biochemistry. Both channel classes are widespread among bacteria and archaea, and there are also examples found in fungi and plants (Pivetti *et al.*, 2003; Haswell and Meyerowitz, 2006). Their role in cell physiology is generally agreed, viz., to facilitate the rapid release of solutes in a nondiscriminating manner, such that cytoplasmic turgor is diminished (Levina *et al.*, 1999). It is frequently observed that bacteria possess examples of both MscS and MscL types and that they are generally functionally redundant (Pivetti *et al.*, 2003). However, the number of organisms in which the roles of the channels has been rigorously tested is limited. The analysis is complicated by the presence of multiple homologues, usually of the MscS class, but occasionally also MscL. Even in *E. coli* their role has only been investigated in laboratory isolates of *E. coli* K-12, where the wall has been weakened by the loss of lipopolysaccharide (LPS).

For both channels, there is an emerging consensus on the structural transitions that they undergo during the opening process. It is given that the channels are closed in the growing bacterial cell and that they undergo rapid structural transitions that create transient large pores 8- to 30-Å diameter. Bacterial cells rely on a selectively permeable membrane to maintain cytoplasmic homeostasis and to interconvert energy via ion gradients (Booth, 1985). The opening of MS channels subverts both of these processes by dispelling ion gradients, lowering the membrane potential, and allowing the nonselective movement of solutes. The lack of selectivity is a function of the large pore diameter that readily allows the passage of solutes probably without the loss of their hydration shell. In the case of the well-characterized Na^+, K^+, and Cl^- channels, ionic specificity is largely attained through the

dehydration of the ion and the replacement of the coordinating water molecules by O and N atoms of the peptide backbone of the channel (Doyle *et al.*, 1998; Zhou *et al.*, 2001; Dutzler *et al.*, 2002). The loss of specificity associated with MS channels is not simply a function of their pore size, since at least MscS homologues exhibit ion selectivity, even if at a more modest scale than observed for classical ion-selective channels (Martinac *et al.*, 1987; Li *et al.*, 2002). The loss of selectivity may be offset by the potentially higher rates of ion permeation, since although some K^+ channels exhibit ion conduction at rates close to the rate of free diffusion, there is a huge dynamic range in the observed conductances.

MS channels in *E. coli* range in conductance from ~0.3 to 3 nS and in patch-clamp analyses they are activated by applied transmembrane (TM) pressure in order of their conductance: MscM, first, followed by MscK, MscS, and finally MscL (Table I; Fig. 1) (Sukharev *et al.*, 1993; Batiza *et al.*, 2002; Kung and Blount, 2004). The structural genes for MscS, MscK, and MscL are known, whereas the gene for MscM remains to be discovered. MscS and MscK are structurally related (see below) but differ in their properties (Levina *et al.*, 1999). In *E. coli*, MscS is an abundant channel activity, whereas MscK is less readily observed in membrane patches.

TABLE I

MscS Homologues in *E. coli*

Protein	Gene	Size (amino acids)	Activity[a]	Expression[b]
MscS	*yggB*	286	Yes (M) (P)	σ^{70}, σ^{38}
F343	*ynaI*	343	No (P)	ND
YbdG	*ybdG*	415	Some (P)	Yes
MscK	*kefA*	1120[c]	Yes (K^+)[d] (M) (P)	LeuO
YjeP	*yjeP*	1101[c]	ND[e]	ND
F786	*ybiO*	741[c]	ND	σ^{38}

[a]Activity can be defined either in terms of activity in membrane patches determined by electrophysiology (M) or by protection (P) afforded against hypoosmotic shock in the triple channel-deficient mutant, MJF465.

[b]Regulation of expression of the gene is reported: σ^{70} refers to the vegetative sigma factor; σ^{38} refers to the stationary phase sigma factor that is also expressed in response to osmotic stress in *E. coli*; LeuO is a general regulatory protein for which the mechanism is not fully understood.

[c]The size includes the signal sequence required for export of the N-terminal domain to the periplasm.

[d]The MscK channel is dependent on high K^+ concentrations on the periplasmic side for activity.

[e]Although not detected in *E. coli*, the growth of YjeP insertion mutants (i.e., null mutants) in *Erwinia chrysanthemi* is inhibited in media containing high K^+ and betaine or proline.

In addition to MscS, five MscS-related proteins are found in *E. coli*. This table summarizes the limited information available for each.

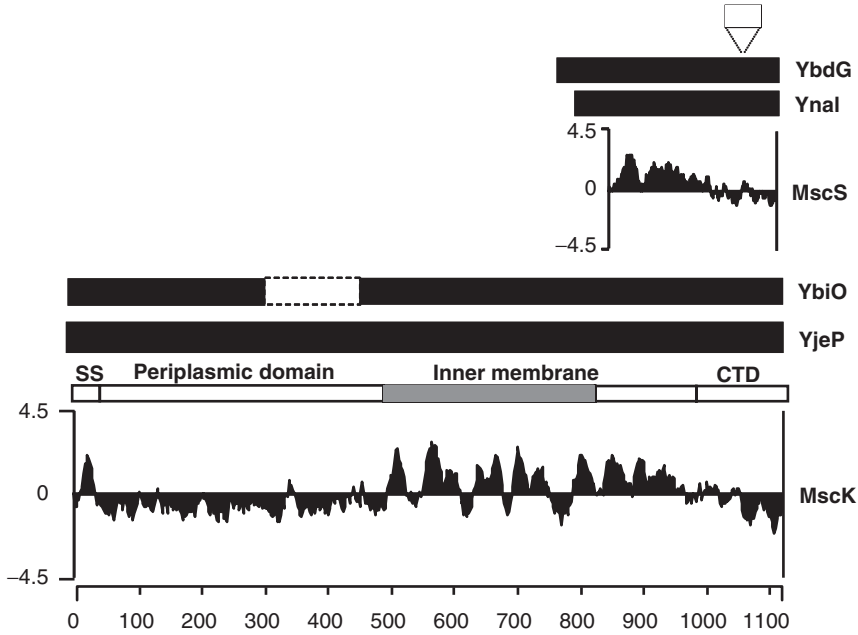

FIGURE 1 The MscS/MscK family in *E. coli*. Hydrophobicity plots for MscS (upper) and MscK (lower) are depicted using a window of 19 residues to define average hydrophibicity at each position using the Protean program (DNAstar). The domain organization of MscK is indicated; note that there are "two" membrane domains—eight helices that lie N-terminal to the "MscS" domain (gray) and the three helices of the "MscS" domain itself. Above each hydrophobicity plot a bar is presented depicting the length of the homologues. For YbiO a gap representing the in-frame deletion in the periplasmic domain that has led to the shorter version of this protein is indicated by a broken line. For YbdG, the insertion that has arisen at the junction between the β- and the $\alpha\beta$-domains is indicated by an open bar connected to the main bar (filled) at the position of the insertion.

MscK opens at pressures just below those needed to activate MscS. MscK also requires K^+ at the periplasmic face for activation and is relatively nonselective for ions (Li *et al.*, 2002), contrasting with MscS, which is variably ion selective (anions in *E. coli* and cations in *Methanococcus jannaschii*) (Martinac *et al.*, 1987; Kloda and Martinac, 2001a,b). Correspondingly, the conductance of MscK is ~0.9 nS and that of MscS is ~1.2 nS (in 0.2-M KCl in the bath and pipette). The two channels differ in that MscS, but not MscK, inactivates under sustained pressure and can only be recovered by resting the membrane patch (Levina *et al.*, 1999). However, in cells MscK appears to have only a minor role in relief of excessive turgor, whereas MscS plays a dominant role in this relief from stress.

A. Functional Overview

MS channels are maintained in the closed state by balanced lateral pressure within the lipid bilayer that prevents the channel protein from expanding to the open state (Perozo *et al.*, 2002a,b). Artificial activation can be achieved by differential intercalation of small amphipaths into one leaflet of the bilayer such that a pressure differential exists between the two halves (Martinac *et al.*, 1990). However, it is generally accepted that *in cells* MS channels gate in response to pressure differentials across the membrane that cause distortion of the bilayer such that cell damage that would result from excessive turgor is avoided. Their function is to release solutes from the cytoplasm and thereby to diminish the TM pressure associated with water influx down the osmotic gradient (Berrier *et al.*, 1992, 1996). Generally, bacteria accumulate solutes to concentrations much higher than the environment leading to water influx and this generates an outwardly directed turgor pressure (Booth *et al.*, 1988). Although precise measurements of turgor pressure are still lacking, the earliest estimates for *E. coli* and *Staphylococcus aureus* of 4 and 20 atm, respectively, remain valid working assumptions (Booth *et al.*, 1988). Analysis of MS channels usually takes place by electrophysiology in isolated membrane patches not protected by the peptidoglycan cell wall (Martinac *et al.*, 1987). Here the most sensitive *E. coli* MS channels, MscM, MscK, and MscS, are generally active at ~0.1-atm pressure applied across the membrane. Clearly the outward turgor pressure in cells is much greater than this value and must be balanced by the resistance of the cell wall (peptidoglycan and LPS, in the case of Gram-negative bacteria; peptidoglycan and lipoteichoic acids in Gram-positive organisms) to maintain the channels closed. Under physiological conditions, MS channel activation occurs in response to a decrease in external osmolarity, which results in an immediate large increase in turgor pressure. Transfer from high osmolarity to low can generate an increase in turgor of up to 10 atm in a few milliseconds, as water rushes into the cell. In these circumstances, the change in the contact between the inner membrane and the peptidoglycan results in membrane distortions sufficient to activate the channels.

As indicated above, channels can also be activated by amphipaths that intercalate into the membrane with a slow transfer time between the outer leaflet and the inner leaflet of the bilayer (Martinac *et al.*, 1987; Perozo *et al.*, 2002b). Such molecular properties could generate transient differences in lateral pressure in the two leaflets leading to distortion-led channel activation. Conceivably, a wide range of molecules could have transient effects on channel gating, for example fermentation products, antibiotics, fatty acids, and so on. Food preservatives, for example parabens (Nguyen *et al.*, 2005) and weak organic acids, and drugs, for example local anaesthetics (Martinac

et al., 1990) that act via the membrane, may be a particular source of transient channel activation. Of perhaps greater significance, periods of cell wall remodeling, particularly in Gram-negative bacteria where a unimolecular layer of peptidoglycan is found, may result in channel activation due to localized changes in tension.

1. Other Functions for MS Channels

To date, the primary function of MS channels has been seen to be relief from the stress associated with hypoosmotic shock—rapid transitions from high-osmolarity environments to low. However, there are many MscS/MscK homologues in *E. coli* (and in many of other organisms) that cannot readily be assayed either as cellular functions or as electrical activities in patch clamp. Conceivably, these proteins have evolved different functions; however, equally, the possession of a gene by an organism does not immediately imply a function within the physiology of that organism. For example, the YjeP, MscK homologue, can be deleted from *E. coli* without any apparent physiological consequence; however, a null mutant in *Erwinia chrysanthemi* leads to sensitivity to the osmoprotectant betaine when cells are grown at high osmolarity in the presence of KCl (Touze *et al.*, 2001). Clearly, in two related organisms an important function for one homologue has been taken over by other proteins. In *Arabidopsis* MscS homologues are implicated in shape regulation and division of chloroplasts (Haswell and Meyerowitz, 2006). When expressed in *E. coli*, at least one of these MS channels is a functional channel and protects mutants lacking MS channels against hypoosmotic shock. While one cannot ascribe functions to all MS channel proteins, it remains possible that some are involved in more subtle processes than simple stress relief.

2. Expression of MS Channels

Small-scale, but significant, changes in MS channel gene expression have been observed in *E. coli* (Stokes *et al.*, 2003). The increase in expression is generally two- to three-fold and is in response to either increases in osmolarity or entry into stationary phase. Both the *mscL* and *mscS* genes are transcribed from promoters recognized by both σ^{70} and σ^{38}, leading to low levels of transcription during vegetative growth. Enhanced expression takes place during osmotic stress and in stationary phase, two conditions where σ^{38} (RpoS) protein abundance increases and forms an RNA polymerase with modified specificity (Hengge-Aronis *et al.*, 1993; Hengge-Aronis, 1996). One of the smaller MscK homologues in *E. coli*, F783 (*ybiO*), is also known to be regulated by σ^{38} (Schellhorn *et al.*, 1998). Mutants lacking σ^{38} exhibit reduced expression both of the MscS and MscL channel genes (and F783) and mutants in which σ^{38} is stabilized express higher levels of the

channels (Stokes *et al.*, 2003). RpoS mutants exhibit extreme hypoosmotic shock-sensitivity after entry into stationary phase (Stokes *et al.*, 2003). However, since σ^{38} also regulates some enzymes involved in stationary phase cell wall remodeling, the mutant phenotype may arise from either the deficiency in channel genes, or altered cell wall. Not all of the expression pattern seen with MscS can be accounted for by the change of the sigma factor from σ^{70} to σ^{38}. It seems highly probable that other protein factors are important for regulation; changes in DNA topology associated with higher osmolarity may also play a role in regulating expression. In the case of MscK, it has been shown that its expression can be lowered by inactivating the LeuO protein, which is considered to be a DNA-binding protein of low specificity (Klauck *et al.*, 1997).

In addition to transcriptional regulation, both *mscL* and *mscS* mRNA molecules have relatively weak ribosome-binding sites with the effect that their translation can be diminished even when transcription takes place. In *E. coli*, and possibly other bacteria, the rate of translation of the mRNA for any protein is determined to a significant extent by the availability of ribosomes and the strength of the ribosome-binding site. The presence of highly abundant mRNA molecules that have strong ribosome-binding sites can cause a significant reduction in the translation of less abundant mRNA molecules with weak ribosome-binding sites. One consequence of this for the MS channels may be to cause a strong degree of heterogeneity in the bacterial population with regard to the abundance of assembled channels. A stochastic distribution of channel subunits would lead to some cells with very few channels since a single channel requires five (MscL) or seven (MscS) subunits. The possession of two independently expressed channels may be a prerequisite for survival of hypoosmotic stress in cells subject to stochastic distribution of the number of subunits, since redundancy reduces the chance of any one cell having no channels.

3. MS Channel Function in Other Bacteria

Lactococcus lactis is one of the few Gram-positive organisms in which the functional role of MS channels has been investigated (Folgering *et al.*, 2005). The genes encoding the MscL (MscL-Ll) and MscS (MscS-Ll) proteins were cloned and expressed in *E. coli* MJF465, which lacks the three major *E. coli* MS channels, MscL, MscS, and MscK. Expression of the *L. lactis* channels protected cells against hypoosmotic shock, indicating retention of function when expressed in *E. coli*. As expected, both channels gave electrophysiological signatures similar to their respective *E. coli* homologues. However, MscS-Ll activity was not detected after fusion of *L. lactis* membranes with liposomes, whereas MscL-Ll activity was readily observed. RT-PCR experiments verified that both *mscL-Ll* and *mscS-Ll* genes were transcribed,

suggesting possible posttranscriptional regulation of MscS protein production. Inactivation of MscL-Ll led to a reduced rate of betaine efflux in response to hypoosmotic shock, but only small changes in survival of the mutant were observed. Since *L. lactis* grows in chains, the authors suggested that this growth morphology would lead to an overestimate of the number of survivors since only one cell in a chain was required to survive to allow a colony to form. An equally plausible explanation is that the incubation conditions predispose the cells to survive despite the inactivation of MS channels. Thus, in *E. coli* a variable fraction of cells of a triple channel mutant (lacking MscL, MscS, and MscK) survive depending on the precise growth conditions. Most significantly, the inclusion of betaine in the growth medium enhances survival (N. R. Stokes, W. Bartlett, and I. R. Booth, unpublished data) and this osmoprotectant was used in the *L. lactis* experiments. Similar influences of betaine may account for the failure of $MscS^{-}$, $MscL^{-}$ double mutants of *Corynebacterium glutamicum* to exhibit significant changes in phenotype (Ruffert *et al.*, 1999; Nottebrock *et al.*, 2003).

III. THE STRUCTURE OF MscS

The crystal structure of the MscS protein was determined at 3.9 Å by Doug Rees's group in 2002 using the *E. coli* protein (Fig. 2) (Bass *et al.*, 2002). Possessing the structure of the *E. coli* protein has generated a considerable advantage for the study of this channel since almost all of the genetics, molecular biology, and electrophysiology had already been conducted with this species. *E. coli* MscS is a 286-amino acid protein and is one of the smallest homologues in this family of proteins. Almost the whole protein is visible in the crystal structure, with only the first 26 and the last 6 residues not resolved. Thus, the crystal structure represents an almost complete image of the protein (Bass *et al.*, 2002). The channel is a homoheptamer with a central pore. The protein falls into two quite distinct domains, a three helix membrane domain and a large cytoplasmic domain. Although there are three helices, only two are genuinely TM, TM1 and TM2. The third helix lines the pore, but in fact only spans the region that is approximately equivalent to the inner leaflet of the lipid bilayer, the rest of the pore is made up from an extended linker that joins TM2 to TM3 and the outer mouth is formed from the N-terminal region of TM1 (Fig. 2). The pore-lining TM3 helix is considered to be in two halves: TM3A and TM3B, with the former lining the pore and the latter being an amphipathic helix that lies along the membrane surface at the junction between the membrane domain and the cytoplasmic domain (Fig. 2B).

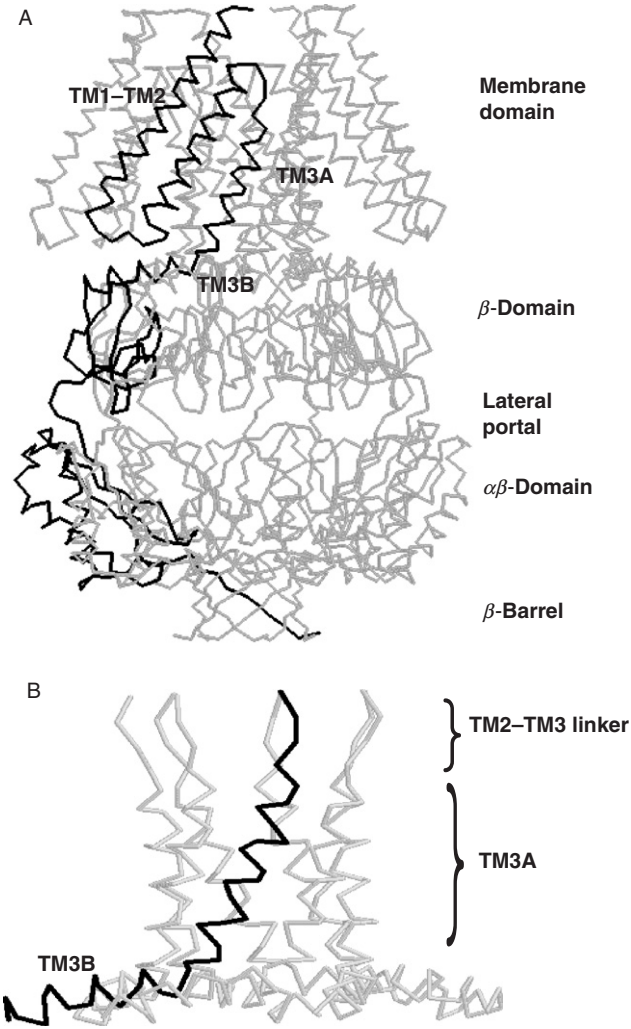

FIGURE 2 The crystal structure of MscS. (A) The heptameric structure is depicted showing only the backbone of each subunit. One subunit is indicated in black to show the path followed by a single subunit. Specific domains referred to in the text are labeled. (B) The structure of the pore region of MscS showing only the path of the TM2–TM3 extended linker and the TM3A and TM3B helices. Again a single strand has been depicted. The images were created using Protein Explorer (Martz, 2002).

A. The Membrane Domain

The membrane domain constitutes only ~40% of the total protein, but can be much more in some homologues that have between 4 and 11 TM spans in all (Fig. 1) (Levina *et al.*, 1999; Pivetti *et al.*, 2003). The *E. coli* MscS protein has three helices with an overall orientation N_{OUT}–C_{IN}, where OUT refers to the periplasmic face of the membrane and IN to the cytoplasmic face (Miller *et al.*, 2003a). TM1 and TM2 pack against each other, but in the crystal state these two helices lie well-separated from the TM3 pore-lining helices and are slightly twisted relative to the axis of the pore (Bass *et al.*, 2002). This conformation is unlikely to reflect the natural state of the channel in the membrane where the lipids will exert pressure to compact the helices such that they make direct contact with the outside of TM3. The removal of the lipid by detergent to facilitate crystallization is principally responsible for the observed conformation, but the formation of stable protein-protein contacts in the crystal may also affect the observed organization. In the crystal structure, the densities for TM1 and TM2 are less well-defined than for TM3, suggesting that they retain either some mobility in the crystal or a number of slightly different alternative packing arrangements (Bass *et al.*, 2002).

The strongest conservation in MscS is TM3, but even here there is considerable diversity among the 19 subfamilies of MscS homologues (Bass *et al.*, 2002; Pivetti *et al.*, 2003). In the crystal structure, this is also the best region of well-defined density. TM3 is considered to consist of two domains: TM3A, residues 96–112 line the pore and TM3B, residues 114–127 form a helix that lies along the surface of the inner leaflet of the membrane and oriented so that it is tangential to the axis of the pore (Fig. 2B) (Bass *et al.*, 2002). Residues 112 and 113, asparagine (Asn) and glycine (Gly) in *E. coli* MscS, act as a hinge allowing the helix to bend. It is notable that while the Asn residue is moderately highly conserved, a number of different residues replace the MscS Gly113 in other homologues. TM3A and TM3B residues define key attributes of the channels, particularly gating pressure, open dwell time, and inactivation kinetics.

B. The Cytoplasmic Domain

The C-terminal domain hangs below the membrane domain, resembling a Chinese lantern—there is a large vestibule created from the seven subunits that is perforated by lateral portals at the subunit interfaces and an axial portal. The domain is suspended from TM3B and this structure may be critical to transmitting conformational changes to the C-terminal domain

(Fig. 2). Each C-terminal domain is an ~17-kDa unit that consists of three subdomains: β (132–177), $\alpha\beta$ (188–265), and the β-barrel (271–280) (Fig. 1). In the channel, the oligomer of the seven C-terminal domains creates a large vestibule that has an external diameter ~80 Å and which is ~70-Å long (Bass *et al.*, 2002). The enclosed chamber of the vestibule varies in diameter narrowing from the portal region (50 Å) to the neck (~27 Å). Access to the vestibule is via seven lateral portals, each ~14-Å wide, created by the junctions between the subunits at the interface between the upper β-domain and the lower $\alpha\beta$-domain. In essence, the β-domain lies immediately below the TM3B segment of the pore-lining helix and narrows the upper part of the vestibule such that there is in effect a wide neck to the cytoplasmic entrance to the pore. The β-domains are themselves a recognized structural fold, the sm-fold, associated with some classes of nucleoproteins, where they form rings around DNA. Usually, these proteins are heptameric but other oligomeric states are possible (Toro *et al.*, 2002).

The $\alpha\beta$-domains combine to form the bottom of the vestibule. The seven-strand β-barrel created by residues 271–280 represents a potential eighth axial portal. However, the diameter is only ~8 Å and its interior is lined with hydrophobic residues and this may prevent easy passage of hydrated solutes. We have shown that the β-barrel may be required for stable assembly of the channel—moreover, the β-barrel is an important structural element that is required for some of the transitions undergone by MscS channels (see below) (Schumann *et al.*, 2004). Small proteins (e.g., GFP and alkaline phosphatase) can be fused to the C-terminus of *E. coli* MscS without severely impairing assembly or gating of the channel (unpublished data). Some homologues naturally have large domains attached to the C-terminus of their MscS protein sequence.

C. Variations in Structure

Despite some specific variations at the C-terminus, this end of the protein tends to be relatively conserved for length. In contrast, MscS homologues with large extensions at the N-terminus are common, with MscK representing a particularly extreme case (Fig. 1) (Levina *et al.*, 1999). MscK is 1120 amino acids (~120 kDa) and has an MscS-like domain at the C-terminus. Immediately N-terminal to this channel-forming domain is a membrane region that has been proposed to form a further eight TM spans (i.e., making 11 in all). At the N-terminus of the predicted protein is a signal sequence that is processed when the protein is exported to the periplasm. This signal sequence ensures that a large (~45 kDa) domain is located to the periplasm. Little or nothing is known about this domain. Constructs that try to recreate

"MscS" from MscK domains are at best poorly active and require addition of a signal sequence for them to be correctly assembled in the membrane (C. Li and I. R. Booth, unpublished data). Thus, whereas *E. coli* MscS can readily achieve an N_{OUT}–C_{IN} organization, the equivalent MscK domain requires either a signal sequence or the rest of the protein to achieve the correct organization in the membrane.

D. Twisting MscS Around the Pore

One of the most important characteristics of MscS is the path followed by the individual subunits relative to the pore (Bass *et al.*, 2002). Each subunit has its N-terminus in the periplasm. The TM3A helices cross the membrane at ~27° and pack tightly against each other with crossing angles of ~22°. On leaving the membrane, the TM3B helix takes a path tangential to the axis of the pore (Fig. 2), and subsequent packing of the β- and $\alpha\beta$-domains causes the path of the subunits to twist around the axis of the channel, a process that is completed by the packing of the protein into the seven-stranded β-barrel. As a consequence of this packing arrangement, the C-terminus of a strand exiting the β-barrel is located ~250°–270° relative to the N-terminus of TM1.

The effect of twisting each subunit around the axis of the pore is probably critical for stability, but even more significant for structural transitions during gating (Edwards *et al.*, 2004, 2005). Indeed it is one of the major properties of MscS that the protein spontaneously oligomerizes when freed from the membrane with detergents (R. Bass, personal communication; S. Miller and I. R. Booth, unpublished data). Given that the protein is stable as a heptamer of free monomers in the membrane, it is inferred from these observations that removal of the lateral pressure generated by the lipid bilayer, allows MscS to adopt alternative packing arrangements from those found in the closed state in the membrane. This process can be accelerated by cross-linking introduced cysteine (Cys) residues.

Uniquely, an S267C mutant forms SDS-stable oligomers, up to and including the heptamer, when cross-linked with the fixed-length reagent *o*-phenylenedimaleimide (*o*-PDM) (Miller *et al.*, 2003b). In rapid succession, dimers are supplemented by trimers through to the heptamer. Other Cys residues inserted close to the position of S267 in the crystal structure do not generate these stable oligomers, despite forming the initial dimer. Placing a Cys residue in MscK at the equivalent position to S267C also generates SDS-stable oligomers (C. Li, S. Miller, and I. R. Booth, unpublished data). This suggests that the property displayed when cross-linked is intrinsic to the structural organization of the proteins. Analysis of the cross-linked proteins

revealed no further cross-links and the multiple forms persisted during purification with gentle detergents. The most remarkable observation is that the trimer, which can only covalently link two of the subunits, carries the third subunit into the SDS-stable state.

E. MscS Is Small but Beautifully Formed

MscS in *E. coli* is a small protein and appears to require all of the elements evident in the sequence and structure. Trimming the *E. coli* MscS protein by making structured deletions affects the stability of the assembled complex (Miller *et al.*, 2003a; Schumann *et al.*, 2004). Removal of the nonconserved stretch from residue 8 to 12 at the N-terminus causes reduced accumulation of the MscS protein in the membrane. Larger deletions (removing residues 8–21) destabilize the protein resulting in the accumulation in the membrane of a truncated protein of ~17 kDa, which may be the C-terminal domain (Miller *et al.*, 2003a). Extending the deletions into TM1 causes almost complete loss of the protein from the membrane. Similarly, the protein does not readily tolerate deletions from the C-terminal end. Proteins that have the base of the vestibule and the β-barrel deleted are less stable than the parent, but larger deletions to the boundaries of the $\alpha\beta$- and β-domains do not result in any active protein and no accumulation of protein in the membrane. The mutants that have the base of the vestibule, including the β-barrel, deleted (Δ266–286) are particularly interesting for function analysis.

The assembled Δ266–286 channels were functional as indicated by their ability to protect a channel-less mutant *E. coli* strain against hypoosmotic shock (Schumann *et al.*, 2004). Further, the channels could be gated by pressure in isolated membrane patches, but the pressure required to gate the channel was slightly higher than that observed for the wild-type channel. However, the significant change was observed after the channels were allowed to undergo desensitization (inactivation). MscS channels, uniquely among MS channels analyzed to date, exhibit the desensitization property. After being maintained open at high, subsaturating pressure (i.e., the pressure required to open multiple channels in a patch and assumed to open all MscS channels present but not great enough to open MscL channels) the channels close. Channel closure follows essentially first order kinetics and the rate is inversely proportional to the pressure on the patch (Akitake *et al.*, 2005). However, this inactive state can be readily distinguished from the closed state. The latter is observed at lower pressures when channels undergo frequent openings followed, a few hundred milliseconds later, by spontaneous closure. Many cycles of opening and closure can be sustained without any apparent loss in channel function. The desensitized state is characterized

by the fact that the patch must be rested at zero pressure for several minutes before channels will again respond to a change in pressure by opening (Koprowski and Kubalski, 1998; Akitake *et al.*, 2005; Grajkowski *et al.*, 2005). We observed that removal of the β-barrel and the base of the vestibule did not affect desensitization per se (Schumann *et al.*, 2004). However, desensitized channels were impaired in their recovery of the active state even after rest for several minutes. The Δ266–286 protein was most affected. The ability to recover could partially be restored by introduction of a sequence of eight amino acids that derive from the histidine (His)-tag. Thus, one can argue that the base of the vestibule, including the β-barrel, is critical for recovery from the desensitized state, but is not otherwise essential. Consistent with the known stability of β-barrel structures, the presence of this feature in MscS may aid correct assembly and facilitate some structural transitions.

Finally, the introduction of Cys residues into MscS must be undertaken with great care to avoid disruption of the structure. In creating Cys-containing proteins, we have observed that despite the cytoplasmic location of the substituted residues, oxidation to form cross-linked proteins frequently occurs. This is not true for all residues (e.g., S267C and S196C are both exempted from this observation), but similar observations have been made for Cys residues located, respectively, on the surface and on the inside of the vestibule. Commonly the oxidized proteins are observed to locate poorly to the membrane and in some cases can only be observed to accumulate if cells are grown in the presence of a reducing agent during the period of induction of expression of the mutant proteins. It seems possible that the Cys residues may oxidize during assembly of the channel protein and that this leads to aberrant conformations that are then subject to degradation. The severity of this effect is position specific and does not generally debar making X to C mutations, merely requiring greater caution than is possibly the case with other membrane proteins.

IV. MscS MUTATIONAL ANALYSIS

The discovery of the *yggB* gene that encodes MscS was a consequence of the analysis of a gain-of-function (GOF) mutation in the *kefA* gene that was subsequently shown to encode MscK (Levina *et al.*, 1999; McLaggan *et al.*, 2002). This mutation affected the ability of cells to grow at high osmolarity in the presence of 0.6-M K^+ and betaine or proline, as osmoprotectants. The mutation was subsequently shown to reside in the TM helix equivalent to TM3A of MscS and altered the gating of the channel, rendering it inappropriately active. It is believed that the mutant channel activates only in the

presence of betaine or proline and high K^+, because under these conditions the cell needs to release K^+ and the normal K^+ efflux systems are blocked. The rise in turgor associated with betaine accumulation precipitates premature channel activation. However, the phenotype of this mutant is critically dependent on the expression level, since placing the mutation (G922S) in the cloned *kefA* gene, which elevates expression 20- to 30-fold, causes growth inhibition even at low osmolarity (C. Li and I. R. Booth, unpublished data), whereas similar expression of the wild-type protein is tolerated.

The discovery of the structural gene for MscS precipitated a flurry of analysis, leading to demonstration that *yggB* gene alone was sufficient to generate channels with the known properties of MscS (Okada *et al.*, 2002; Sukharev, 2002), the crystal structure of the protein, and the search for mutants. The equivalent search for *mscL* gating mutants had been highly successful, simply by screening for growth defects associated with expression of *mscL* genes that had been mutagenized (Blount *et al.*, 1996a,b, 1997). An equivalent analysis of MscS mutants yielded only a single mutant allele, V40D, expression of which blocked growth, accelerated K^+ loss, and rendered cells sensitive to hypoosmotic acid shock (Okada *et al.*, 2002). The failure to find other MscS GOF mutants may arise from the abundance of this channel since many GOF alleles were isolated in MscK, which is expressed at lower levels, and could be constructed by site-directed mutagenesis in MscS when suitable precautions are taken (Miller *et al.*, 2003a; Edwards *et al.*, 2005).

Interesting mutations that modify the gating of MscK were a product of a screen in *Salmonella typhimurium* for mutations that would allow a *nadB* mutant strain to grow on 0.1-mM quinolinic acid (QA). Normally *nadB* mutants require 10-mM QA for growth, a phenotype that is believed to arise either from poor entry of the acid or rapid expulsion. Among the mutants allowing growth at 0.1-mM QA were five *kefA* (*mscK*) alleles: R792P, L866Q, W909R, A918P, and G924S. The first two mutations are outside the TM3A pore-forming helix, but the other three are either in the sequence equivalent to TM3A or in the extended loop connecting TM2 to TM3A. Transfer of the mutations to their equivalent positions in MscS (A918 and G924 are conserved residues) generated GOF phenotypes indicating functional equivalence between the pore structures in MscS and MscK. Similarly, creating T93R in MscS, the equivalent of W909R in MscK, also generated a GOF phenotype which was the first indication of the importance of this sequence in the gating transition (Miller *et al.*, 2003a). The other two alleles are also interesting since both are less severe GOF alleles in MscK and R792 is not represented in the MscS structure since it forms part of the linker that connects the "MscS domain" to the rest of the MscK protein. L866Q, when recreated in MscS, does not have a strong phenotype and this reveals

potentially significant differences in the two structures. In MscK, L866Q is a mild GOF allele, whereas a double mutation in MscS, close to the equivalent position (I48D, S49P; single mutations have no observable phenotype) blocks gating. Subsequent studies have created many mutations in both MscS and MscK that have facilitated model building for the gating transition. However, it is frequently observed that introducing mutations into MscS destabilizes the protein and thus the absence of observable mutants in the more generic growth-inhibition screens may arise from the significant structural perturbation such mutations generate.

V. STRUCTURAL TRANSITIONS IN MscS

A. The Need for the Closed State

Bacterial cytoplasmic membranes are simultaneously the site of energy transduction and the location for MS channels. The opening of the latter will depolarize the membrane and will perturb cytoplasmic ion pools leading to a loss of homeostasis and diminished energy production. Consequently, MS channels must remain in the closed state for much of the time and after opening they must revert to the closed state quickly to avoid impairing the growth (and survival) of the cell. This has formed the basis for the selection of gating mutants in MscL and in a more limited sense for MscS mutants (Blount *et al.*, 1996b, 1997; Okada *et al.*, 2002). In both cases, growth inhibition results from expression of channels that gate more readily at lower pressure than the wild type. However, the correlation is not straightforward. Growth inhibition is the product of the expression level of the protein, its stability in the membrane and the effect of the actual amino acid change on both the threshold pressure for channel activation *and* the open dwell time. A mutant that gates at lower pressure, but which also either affects channel assembly or the open dwell time of the channels, may not inhibit growth of the bacterial cell. This is exemplified by the N15D MscL mutant (Buurman *et al.*, 2004). At the low levels achieved by expression from the chromosome, the mutant channels facilitate growth at low K^+ concentrations of a mutant strain lacking the normal K^+ uptake systems, that is, N15D activity improves cell physiology because of the particular problems of this *E. coli* mutant strain. In contrast, expression of the same mutation from a high copy plasmid has a very severe effect on growth (Ou *et al.*, 1998). Similarly, we have often observed compensation arising from simultaneous changes in open dwell time and threshold pressure for activation, with one effect offsetting the other.

In both MscL and MscS, the ion impermeability of the closed state of the channel is maintained by rings of hydrophobic residues. In the case of MscS,

these are two rings of leucine (Leu) residue, L105 and L109 (Bass *et al.*, 2002). L109 lies immediately adjacent to the cytoplasmic neck of the pore, with L105 residues in a ring immediately above this (i.e., toward the periplasmic face)—thus the seal is not symmetrically located at the middle of the membrane, but lies closer to the cytoplasmic face. This feature may be critical to the gating transition (see below). Substitution of small residues or hydrophilic residues at positions 105 and 109 creates channels that gate at lower pressures. The greatest effects are seen at position 105. However, the channels are closed until pressure is applied, contrary to speculation that such channels would be open pores (Edwards *et al.*, 2005). Insertion of larger hydrophobic residues in place of Leu creates stable channels, but these have a tendency to require higher pressure for gating.

B. The Crystal State

In the crystal form, MscS is a homoheptamer that has been trapped in an open state. A central pore is seen down the long axis of the protein. The observed diameter of the pore at 8–11Å (depending of the method of assessment) is smaller than predicted (14 Å) from conductance measurements for the fully open channel (Sukharev, 2002). This has led to a degree of controversy (Anishkin and Sukharev, 2004) concerning whether this protein is the open state, the closed state, or "an open state." The latter represents a compromise between the two extreme states that the channel could occupy. Whatever state the structure represents, it is clear that a hydrated ion or low-molecular-weight solute could pass through the pore as displayed in the crystal form. Other biochemical evidence suggests that the closed form of the channel is more compact than that depicted in the crystal structure (Miller *et al.*, 2003b). Thus, we have shown that single Cys residues substituted for serine (Ser) (there are no endogenous Cys residues in *E. coli* MscS) that are greater than 10 Å apart in the crystal structure can readily be oxidized by Cu/phenanthroline reagent. The most significant of these data were derived using studies of two mutants: S58C and S267C residues, which are located at the base of TM2 in the membrane domain and at the bottom of the vestibule in the C-terminal domain, respectively. In adjacent MscS subunits, these residues are separated in the crystal structure by ~19 (S267C) and 33 Å (S58C). The residues were rapidly cross-linked. Bringing S58C residues together could be achieved by packing the TM1–TM2 helices closer together against TM3; however, significant mobility would still be required to facilitate the formation of the cross-link since TM1 helices would be placed between TM2 in such a packed structure. Perhaps more significantly for the compact closed structure model, S267C residues are buried in the

crystal state, such that neither residue should easily react with the next one (Fig. 3). However, in the closed state, that is, the channel embedded in the membrane, the residues readily react and are cross-linked by Cu/phenanthroline and MTS-1-MTS. Iodine cannot be used with any confidence as an oxidizing agent to study MscS, as incubation with this compound has been found to cause the rapid degradation of the protein, even in the native protein where Cys residues are absent (S. Miller, unpublished data). These data, plus other recent unpublished studies, point to the closed channel being in a compact state relative to that depicted in the crystal structure.

Other data support a more compact form for the closed state of the channel (Koprowski and Kubalski, 2003; Grajkowski *et al.*, 2005). Cross-linking lysine (Lys) residues by 1-min exposure of membrane patches to bis (sulfosuccinimidyl)-suberate caused loss of channel activity that could not be reversed by washing out the cross-linking reagent. Since all except one Lys residue are situated in the cytoplasmic domain, these data were interpreted to indicate that preventing C-terminal domain movement blocked channel gating (Koprowski and Kubalski, 2003). In the same study, Kubalski and colleagues demonstrated that Ni^{2+} could block the transition from the closed to the open channel when added to membrane patches from cells expressing MscS protein with a C-terminal His_6-tag. The effect could be

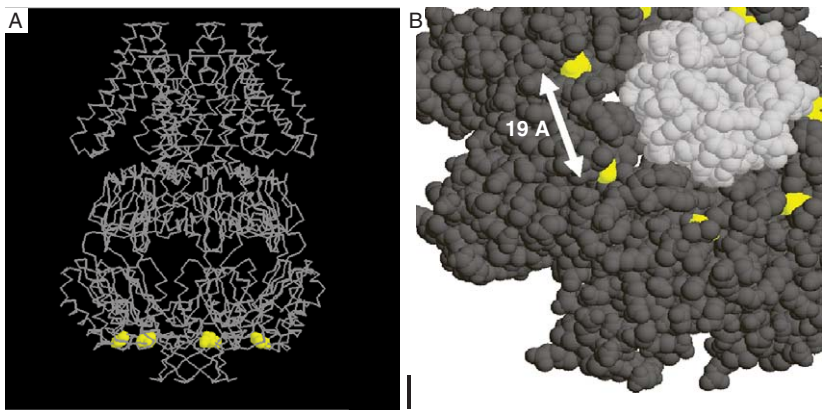

FIGURE 3 The position of the S267 residue. The position of the S267 residue, which when modified to S267C and cross-linked with *o*-PDM leads to SDS-stable oligomers, is indicated. (A) The position of the residue relative to the whole channel protein is indicated by a space-filled residue (yellow) against the backbone of the subunits. (B) A space-filled model viewed from the base, in which the base of the vestibule is dark gray, the β-barrel is pale gray, and the S267 residues are yellow. Images were created using Protein Explorer (Martz, 2002).

reversed by washout of the Ni^{2+} and was not seen when the channel lacked the inserted C-terminal His_6-tag. Moreover, this group also demonstrated that addition of high-molecular-weight ficoll [400 kDa; 1–10% (w/v), final concentration] to the bath increased the rate of inactivation of the channel and diminished the total number of active channels (Grajkowski *et al.*, 2005). The presence of ficoll increased the pressure required to activate the channels. Addition of ficoll to the periplasmic side of the patch slowed channel inactivation but was without effect on the number of active channels. Clearly, these data indicate a greater effect of ficoll from the cytoplasmic side of the patch, that is, action via the C-terminal domain. The high molecular mass of the ficoll would probably prevent it entering the vestibule and consequently the effects have been interpreted as arising from inhibition of structural transitions in the C-terminal domain that are required for closed-to-open transitions in MscS. These data are consistent with the MscS protein making a large conformational change during the transition from the closed to the open state, which is consistent with the crystal structure representing one open state that the protein can achieve.

C. The TM3 Pore

One of the most obvious features from the structure of *E. coli* MscS is the very tight packing of the TM3A helices, which are in the closest proximity possible (Bass *et al.*, 2002; Edwards *et al.*, 2005). This is due to the conservation of Gly and alanine (Ala) residues such that the former creates a surface against which the Ala residues are packed. The *E. coli* MscS family of homologues carries the sequence $A_{98}hhG_{101}A_{102}hG_{104}hA_{106}hG_{108}hA_{110}hyG_{113}$, where A and G have their normal single letter code meaning, h = hydrophobic and hy = hydrophilic residues. In the crystal structure, A106 and G108 are within van der Waals radii of each other, but A98–G101 and A102–G104 are packed somewhat more loosely. The helices cross each other at an angle of 22° (Bass *et al.*, 2002). We proposed that the Ala residues formed knobs that slid across the grooves created by the Gly residues in the adjacent helix and that the closed-to-open transition involved rotation of the TM3A helix such that new contacts were established between different Gly and Ala pairs (Edwards *et al.*, 2005). This hypothesis was tested by changing Ala to valine and Gly to Ala to create proteins in which bulkier residues replaced the simple knobs and grooves. All of these mutants proved more difficult to gate. Conversely, the substitution of Ala by Gly removed the knobs and created channels that opened more easily (A106G). A similar mutation further up the pore (A102G) was affected in that its open dwell time was much reduced, suggesting that in this case removing the knob removed the stabilizing factor

for the open state. A double mutant that recreated Ala-Gly packing but on opposite helices (i.e., A106G/G108A) exhibited a return to normal gating pressure. These data support the idea that TM3A helices rotate during the closed-to-open transition such that the Ala residues cross over the Gly surfaces. It seems likely that the limit of this structural transition is imposed by bulky hydrophobic residues both in the lumen of the pore (L105 and L109) and in the interfaces packed against the TM1–TM2 pair, but this hypothesis has not been fully tested.

During the course of analyzing the importance of the conserved Gly-Ala packing in MscS, we observed that the effects of mutations was of increasing severity when the changes were made close to the seal of the channel compared with mutations created higher up TM3A (Edwards *et al.*, 2005). For example, A106V displays two open states—an unstable wild-type conductance that is seen at pressures intermediate between those that open MscS and MscL and then a low conductance state that is the dominant form of activity at high pressure equal to those needed to gate MscL. Similarly, G108A, A110V, A106L, A106S, and A106G channels exhibit lowered conductance. Ser residues are strongly perturbing where the path of the helix in the wild-type state is constrained. Ser residues have the capacity to form intrahelical and interhelical H-bonds that can perturb helix path and/or helix packing. Gly to Ser mutations at positions 101, 104, and 108 progressively lower the duration of the open channel from ~250 to 1 ms. This open dwell time analysis points to greater constraints on the helix packing around the seal than was the case higher up the channel pore. Consequently, the model envisaged that to achieve the fully open state the helices would tilt outward to a greater extent at their periplasmic ends than at the pore region. Modeling of the TM3A helices also predicted this structural transition to account for the creation of the pore (Edwards *et al.*, 2005). Given that the known state is an open one (though not necessarily the fully open state) in the crystal form, by reference to this structure one must note that to achieve the closed state the TM3A helices must attain a more vertical state, pack more closely (by moving to Ala98-Gly102 tight packing), and be rotated such that their Leu residues point toward the center of the channel pore.

D. The Closed-to-Open Transition

High-resolution recordings of MscS channel activity have indicated that the channel may open via an ion conducting substate that is short-lived (~20 μs) and which has a conductance 2/3 of the fully open conductance (Shapovalov and Lester, 2004). This contrasts with MscL where many substates are seen and some of the GOF mutants lead to higher occupancy

of some of the subconducting states (Anishkin *et al.*, 2005). No comparable analysis has been completed for MscS GOF mutants, although as referred to above, we have observed some GOF and loss-of-function mutants to exhibit lowered conductance. MscS displays the potential to be voltage-gated and the capacity for inactivation. These two issues frame the discussion of the closed-to-open transition.

Several charged residues reside in the TM1 and TM2 helices (R46, R74, and R88) and the structure resembles a voltage sensor (Bass *et al.*, 2002). This led to the proposal that the channel was voltage and pressure sensitive, in line with earlier observations of channel activity in membrane patches (Martinac *et al.*, 1987). Subsequent analysis has indicated that MscS activation is essentially voltage independent but that the inactivation process may be voltage sensitive (Akitake *et al.*, 2005). At high negative holding potentials (negative patch pipette voltage), the rate of inactivation of channels was enhanced. If, as expected, the TM1–TM2 sensor paddle is able to move in the electrical field, then application of a TM voltage (pipette negative) could cause a significant displacement of this part of the channel. Given the importance of the link between TM2 and TM3 for channel activation, it is reasonable to expect that these conformational changes would be transmitted to the pore.

Clearly, these phenomena are important aspects of the structural transitions that MscS can undergo in patches, but are they relevant in the context of the cell? Bacterial cells have membrane potentials varying between −60 and −240 mV (inside negative), depending on the organism and the environmental conditions. The polarity of the field is, however, more significant than the dimensions with respect to the activation/inactivation of MscS. In the normal state, the membrane potential is negative inside (i.e., positive outside) which is the opposite of the polarity applied in patch clamp to effect changes in the inactivation rate. Perhaps more significantly, MscS is almost certainly the third MS channel in *E. coli* to open, since the sequence observed in patches is MscM, followed by MscK and then MscS and finally MscL (Batiza *et al.*, 2002). The current-carrying capacity of either MscM or MscK should be sufficient to depolarize the membrane such that when MscS is open there is no significant potential to affect the kinetics of the channel. Conditions used to measure MscS activity may, therefore, lead to properties that do not have a corresponding cellular dimension. For example, inactivation of MscS channels is seen when high pressure is sustained on the patch for an extended period up to several seconds. However, in cells the action of opening the channels dissipates the pressure gradient and the expected duration of the open state should be in the order of milliseconds rather than seconds. Thus, inactivation may be a measurement artifact rather than an important functional attribute of MscS.

We have proposed a model for the closed-to-open transition based on our cross-linking data and the observations made by other groups (Fig. 4) (Edwards *et al.*, 2004, 2005). In the closed state, we envisage MscS TM3A helices to be closer to the perpendicular than in the crystal structure and consequently to exhibit altered packing between the potential Gly-Ala pairs formed between adjacent TM3A helices. TM1–TM2 pairs are held against TM3A by the lateral pressure within the lipid bilayer. The conformational change in TM3A is transmitted to TM3B such that the packing of the β-domains is modified and this may aid maintenance of the closed state. The overall effect is that both the membrane and the cytoplasmic domains are in a more compact conformation. Distortion of the membrane bilayer allows unpacking of the TM1–TM2 paddle from TM3A and this change is sufficient to allow the pore-lining helices to rotate and tilt such that the pore

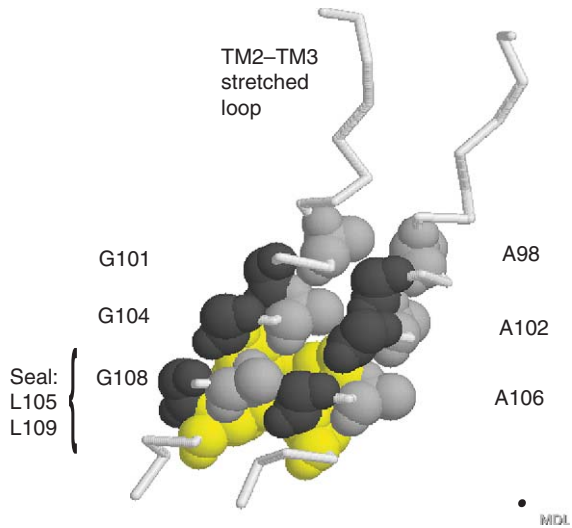

FIGURE 4 The closed-to-open transition in MscS. The backbone of two TM3A helices is depicted with the Gly (dark gray) and Ala (light gray) pairs indicated relative to the position of the Leu seal. The Gly residues provide surfaces over which the Ala residues slide to provide the smooth transition to the open state. In the closed state, A98-G101 and possibly A102 and G104 are proposed to approach each other as the helices turn and straighten. The open state must be stabilized and this may require that the fully open state involves the crossing of bulkier residues to form a resistance to prevent the collapse back to the closed state. In support of such a model it has been observed that Ala to Gly mutations cause channels to become unable to sustain an open state, but that this can be suppressed by mutagenizing Gly to Ala at other positions in TM3A (unpublished data). Images were constructed using Protein Explorer (Martz, 2002).

enlarges. Such molecular motion must be accompanied by increased separation of the helices to create a pore of sufficient size for hydrated ions and small solutes to pass through rapidly. In molecular dynamics simulations of MscS, much has been made of the changes in hydration and the potential for the crystal structure to represent the closed state blocked by a vapor lock (Anishkin and Sukharev, 2004; Spronk *et al.*, 2006). There can be no doubt that opening the pore must be accompanied by changes in water structure close to the surface of the pore but whether the vapor lock is real is unclear (Spronk *et al.*, 2006). TM3A rotation will alter the conformation of TM3B with consequences for the packing of the C-terminal domain, such that the expanded structure seen in the crystal form is generated. However, critical datasets that might allow verification of this model for the gating transition are lacking at present.

VI. CONCLUSIONS AND FUTURE PERSPECTIVE

Like all good models the crystal structure has generated speculation, experimentation, and structured simulations. Not knowing the precise state represented by the crystal structure is a disadvantage, but simultaneously it has narrowed the options on the structural transitions undertaken by MscS. Further structures will be welcome additions to this canon, as will the publication of data from Perozo's laboratory that have used site-directed spin labeling to examine the movements of TM1–TM3 during gating (E. Perozo, personal communication). The analysis of MscL by this method was critical to building a model that is generally accepted for the gating transition (Perozo *et al.*, 2001, 2002a,b). Finally, understanding the structural transitions in MscS lies at one end of the spectrum of our knowledge of this system. Equally important is to go back and place the channel in the context of cell physiology to increase the understanding of the cellular function of this channel and its homologues.

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