CHAPTER 11

The MscS Cytoplasmic Domain and Its Conformational Changes on the Channel Gating

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I. Overview
II. MscL and MscS: Primary Gates and Similarities in Activation
III. The MscL Cytoplasmic Regions and Functioning of the Channel
IV. The MscS C-Terminal Chamber: The Cage-Like Structure and Kinetics
V. Structural Alterations of the MscS Cytoplasmic Chamber on Gating
VI. Conclusions and Perspectives
References

I. OVERVIEW

The cytoplasmic domain of the bacterial mechanosensitive (MS) channel of small conductance (MscS) is shaped by its C-termini forming a large chamber filled with water. Several independent studies indicate that the chamber is a dynamic structure that undergoes severe conformational changes on the channel gating. Various electrophysiological and biochemical methods combined with molecular biology have been used to investigate this phenomenon and the results are presented in the chapter. The size of the chamber and its shape resemble cytoplasmic domains from eukaryotic non-MS channels whose function in stabilization of the channel closed state is established. Analogous role of the MscS cytoplasmic chamber is discussed.

Bacterial MS channels protect these cells against hypoosmotic shock. Two types of MS channels from the cytoplasmic membrane of *Escherichia coli*,

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MscL and MscS (the large and small conductance MS channel, respectively, see also other chapters of the book), play an essential role in the physiology of this bacterium, allowing efflux of solutes from the cytoplasm when osmolarity of the external medium decreases (Ajouz *et al.*, 1998; Levina *et al.*, 1999; Batiza *et al.*, 2002). Homologues of these channels have been found widely in other bacteria (Moe *et al.*, 1998; Levina *et al.*, 1999) and archea (Kloda and Martinac, 2002). Few eukaryotic homologues of both channels have also been identified and they include: structurally related MscL protein from *Neurospora crassa* and putative membrane proteins from *Arabidopsis thaliana*, *Saccharomyces pombe*, and *Drosophila melanogaster* showing homology to MscS (Koprowski and Kubalski, 2001; Pivetti *et al.*, 2003). Two MscS-like proteins from *Arabidopsis thaliana* have been shown to function as channels and to control plastid size, shape, and perhaps division of plant cells during normal development (Haswell and Meyerowitz, 2006). It is not known, at present, if analogous functions can be attributed to the bacterial MS channels.

II. MscL AND MscS: PRIMARY GATES AND SIMILARITIES IN ACTIVATION

The activities of MscL and MscS can be recorded after reconstitution of purified proteins in planar lipid bilayers (Häse *et al.*, 1995; Blount *et al.*, 1996; Okada *et al.*, 2002; Sukharev, 2002), indicating that no auxiliary proteins are necessary for the MS conduction of ions and both channels sense membrane stress directly. Their single channel conductances are large if compared to the conductances of eukaryotic ion channels and are 1 and 3.5 nS for MscS and MscL, respectively. In a typical patch-clamp experiment, MscS is activated at membrane tension of about 5.5 dynes/cm (Sukharev, 2002), whereas for the opening of MscL considerably higher tension must be applied (around 10 dynes/cm) (Chiang *et al.*, 2004). The activities of both channels recorded directly from the *E. coli* membrane are kinetically distinct: MscS opens for hundreds of milliseconds (Martinac *et al.*, 1987), whereas the MscL open-dwell times are in the range of tens of milliseconds (Sukharev *et al.*, 1993). The MscS activation is dependent on the rate of applied stimulus and channels fully respond to the abrupt pressure changes but do not open at those applied slowly (Akitake *et al.*, 2005). MscS shows inactivation during sustained pressure (Koprowski and Kubalski, 1998; Akitake *et al.*, 2005), while MscL does not. Except of being an MS channel, MscS is modulated by voltage (Martinac *et al.*, 1987), and it has been demonstrated that at lower depolarizing voltages (below −40 mV) the channels inactivate easily (Akitake *et al.*, 2005). Both channels are

regulated by pH but in a different way. MscS is completely blocked by pH below 5.5 (Cui *et al.*, 1995), while low pH shifts the MscL activation curve toward higher pressures (Kloda *et al.*, 2006). Comparing gating characteristics of both channels clearly indicated that the basic conformational rearrangements in MscS should be more complex than those in MscL. A release of amino acid sequences first and then solving crystal structures of both channels confirmed this assumption.

The MscL functional channel is a pentamer (Fig. 1B), and each 136-amino acid subunit consists of two α-helical membrane-spanning domains TM1 and TM2 with both C- and N-termini located in the cytoplasm (Sukharev *et al.*, 1994; Blount *et al.*, 1996; Chang *et al.*, 1998). TM1s line the pore and their hydrophobic residues form the transmembrane (TM) gate (Chang *et al.*, 1998; Batiza *et al.*, 1999). The MscL quaternary structure reveals its closed conformation. On the basis of this structure and the analysis of the channel gating, the open conformation has been predicted (Sukharev *et al.*, 2001a,b) and experimentally confirmed (Betanzos *et al.*, 2002; Perozo *et al.*, 2002). MscS is a 286-amino acid protein and its crystal structure (Fig. 1A) reveals that the channel is a heptamer (Bass *et al.*, 2002). Each of seven subunits is composed of three TM helices TM1, TM2, and TM3. The highly conserved TM3 helices rich in glycines and alanines line the channel pore. The cytoplasmic domains of the channel are composed mostly of β-sheets and surround a large water-filled chamber of diameter ~40 Å. Each domain consists of a middle β-domain and a lower α/β-domain (Fig. 1A), and all seven subunits are linked together by a β-barrel composed of seven strands that are located at the very ends of C-termini. The chamber has seven pores, 14 Å in diameter each, located at the subunit interfaces and the additional opening of 8-Å diameter formed by a β-barrel at the bottom of the chamber. It has been suggested that the structure reveals an open channel conformation (Bass *et al.*, 2002) but molecular dynamics studies of water inside the MscS channel implicated that the structure may represent an inactive state of the channel (Anishkin and Sukharev, 2004; Akitake *et al.*, 2005). On the basis of crystallographic data, a closed nonconducting conformation of the channel has been proposed (Bass *et al.*, 2002; Edwards *et al.*, 2005).

Although structurally different both channels, according to the existing models, are activated in a very similar fashion. The gates in both channels in a nonconducting state are formed by a tight constriction rings of hydrophobic residues within the TM domains lining the pore and the opening of the channel involves a tilt and rotation of these domains (Yoshimura *et al.*, 2001; Sukharev *et al.*, 2001b; Betanzos *et al.*, 2002; Perozo *et al.*, 2002; Barlett *et al.*, 2004; Edwards *et al.*, 2005). The rearrangements within MscS are, however, of smaller magnitude than in MscL (see also other chapters of the book). If the structural rearrangements within TM domains lining the

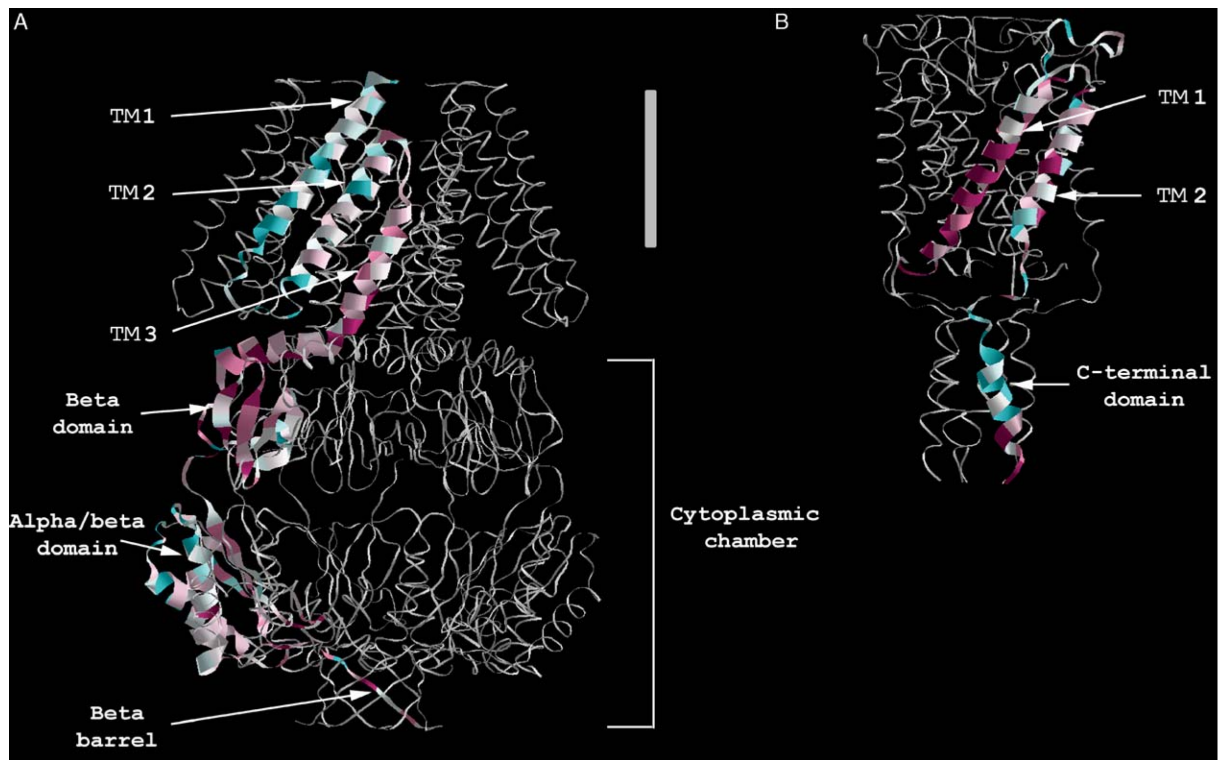

FIGURE 1 Crystal structures of bacterial MS channels of small MscS (A), and large MscL (B) conductance. One subunit of each channel was colored with conservation scores (magenta, conserved; cyan, nonconserved) by the program Consurf (Glaser *et al.*, 2003; Landau *et al.*, 2005) available online at: http://consurf.tau.ac.il/. The gray bar in the middle of the figure represents an approximate thickness of the membrane.

pores in both channels are of similar character, what makes the channels kinetically distinct and which regions of MscS underpin its inactivation? According to the "dashpot" model of the MscS gating, the open conformation of the channel is accomplished by a concerted movement of all three TM helices aside from the vertical axis of the channel (Akitake *et al.*, 2005). A transition from the open to the inactivated state, whose conformation is represented by the crystal structure, involves detachment of the TM3 helices from the TM1–TM2 assembly and their collapse to the closed, nonconductive conformation. The detachment of TM3 is stimulated by depolarizing voltage acting on positive charges located in TM1–TM2 and pushing them toward a position that is perpendicular to the plane of the membrane (Akitake *et al.*, 2005).

III. THE MscL CYTOPLASMIC REGIONS AND FUNCTIONING OF THE CHANNEL

The modeling of the conformational transitions on gating is much more advanced in MscL than in MscS and it includes also, except rearrangements of the TM helices, structural changes and a role of the channel cytoplasmic N- and C-termini. It is postulated that there are two gates involved in the opening of the channel. Except the main TM gate, there is a second cytoplasmic gate (Sukharev *et al.*, 2001a,b) composed of five α-helical S1 segments of the cytoplasmic N-termini (not resolved in the crystal structure and not indicated in Fig. 1B) acting in accordance. The TM gate is proposed to act as a pressure sensor and on application of pressure; this gate permits initial expansion of the channel without its full opening (Betanzos *et al.*, 2002; Sukharev *et al.*, 2005). The cytoplasmic gate that allows full activation of the channel is being connected with TM1s via flexible linkers. According to the model, the applied pressure is transmitted to the S1 segments through the flexible linkers and pulls them apart. The channel may fully open when the interactions between five S1 segments of the cytoplasmic gate break down.

The cytoplasmic C-terminal domain of MscL is formed by a bundle of five helices (Fig. 1B) connected to the TM2 by linkers containing clusters of charged residues RKKEE. According to the present model (Anishkin *et al.*, 2003), the bundle remains stably associated on transition from the closed to open conformation of the channel, serving as a size-exclusion filter. The investigation of the role of charged cluster revealed, however, that it functions as a proton sensor adjusting the channel sensitivity to membrane tension in a pH-dependent manner and, therefore, having an influence on the channel gating (Kloda *et al.*, 2006).

IV. THE MscS C-TERMINAL CHAMBER: THE CAGE-LIKE STRUCTURE AND KINETICS

On the basis of available experimental data, the current models of the MscS gating focus mainly on the function of the TM domains, and at present the contribution of the large cage-like structure formed by its C-termini is not clearly assessed. The size of the structure and its complexity create main obstacle in its investigation and a subsequent modeling. There is, however, a quite large body of evidence that the MscS large cytoplasmic chamber, unlike that of MscL, is a dynamic structure changing its shape on the channel gating and essential for structural transitions undergone by the channel.

The cytoplasmic domain is composed mostly of β-sheets and surrounds the large water-filled chamber of diameter ~40 Å. Each of seven subunits of the assembly consists of a middle β-domain and a lower $\alpha\beta_3\alpha$-domain and all of them are linked together by a β-barrel composed of seven strands that are located at the very ends of C-termini. The MscS cytoplasmic chamber resembles "hanging gondola-like" structure of the cytoplasmic T1 domain of the eukaryotic voltage-dependent potassium channels (Sokolova *et al.*, 2001; Kobertz *et al.*, 2002) or that described for the acetylcholine receptor (Miyazawa *et al.*, 1999). It is well documented now that the cytoplasmic domains of eukaryotic potassium channels including inward rectifiers and those activated by voltage, Ca^{2+}, or cyclic nucleotides affect the activity of the associated pores by controlling the ion flow and ultimately providing an additional gate, or by coupling intracellular signals to the channel primary gate (Yi *et al.*, 2001; Roosild *et al.*, 2004). It is of great interest, therefore, to provide an evidence for a possible function of the large cytoplasmic chamber of MscS by investigating its structural rearrangements, link them to the channel gating, and eventually couple to the intracellular signaling pathways. Results obtained independently by different research groups indicate that the MscS cytoplasmic chamber is a flexible structure and it may undergo significant structural rearrangements occurring on the channel transitions from one functional state to another (Koprowski and Kubalski, 2003; Miller *et al.*, 2003; Schumann *et al.*, 2004; Grajkowski *et al.*, 2005).

An observation that in the amino acid sequence of MscS, all lysines but one (K60) are situated in the C-terminus led to device a series of experiments in which the lysines were cross-linked. It was expected that a cross-link of lysines from different C-termini of the channel would hamper or prevent the channel opening, providing its C-termini being pulled apart during opening. A series of patch-clamp experiments was performed in which lysine-specific reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride—EDC and highly lysine-specific bis(sulfosuccinimidyl)suberate—BS^3 were applied to

the cytoplasmic side of MscS in its closed configuration (Koprowski and Kubalski, 2003). Indeed, the cross-link of the MscS C-termini yielded inactive channels and the effect was irreversible. In the same study, it has also been demonstrated that Ni^{2+} coordination in the MscS-His_6 channels (His-tag added at the very end of C-terminus) prevented the channels from opening. The Ni^{2+} coordination leading to the reversible inhibition of activities was observed in the channel closed state but not in the channel open conformation, suggesting that the closed state of the channel is the only configuration in which the intersubunit coordination of Ni^{2+} may occur. It has been suggested that the lack of the effect of Ni^{2+} application to the open MscS-His_6 channels is due to binding of metal ions to histidines within individual poly-histidine tag. This observation provided the first experimental evidence that C-termini may move apart on the channel opening and may be involved in the process of the channel gating.

These data were supported shortly by a biochemical study (Miller *et al.*, 2003) that demonstrated *in situ*—and, accordingly in the closed state of the channel—a cross-link of cysteines substituting serines 267 (indicated in pink in Fig. 2) located at the bottom of $\alpha\beta_3\alpha$-domain of the cytoplasmic chamber. The cysteines were cross-linked by *o*-phenylene-1,2-dimaleimide (*o*-PDM) that cross-links residues that are capable of adopting positions within 10 Å of each other. Strikingly in the crystal structure of MscS that represents open or inactivated channel conformation, the serines 267 are $\sim$20 Å apart. On the basis of these data, the authors suggested large flexibility of the MscS cytoplasmic chamber and proposed a model of the closed state of the channel with an assumption that the crystal structure shows the MscS open configuration. According to the model, the closed, nonconductive state would be represented by a more compact configuration of the cytoplasmic chamber in which an eventual collapse of the entire $\alpha\beta_3\alpha$-domain may lead ultimately to an impermeable conformation of the cytoplasmic structure (Edwards *et al.*, 2004). It is, therefore, suggested that the chamber may represent an additional, flexible permeability filter. The model has been referred to as the "Chinese-lantern" representation by analogy with a lantern, whose light intensity is related to the extent of its expansion. However, this hypothesis needs experimental exploration. The molecular dynamics simulations that tested transitions from the channel conformation revealed by crystallography to the closed and then to the open configuration have shown that the side openings of the C-terminal chamber did not assume a completely closed state in none of the simulations. The bottom opening, however, remained closed (did not conduct water molecules) even after the open state of the channel was imposed by application of surface tension (Sotomayor and Schulten, 2004). Another study, in which small cosolvents exerted a different effect on the

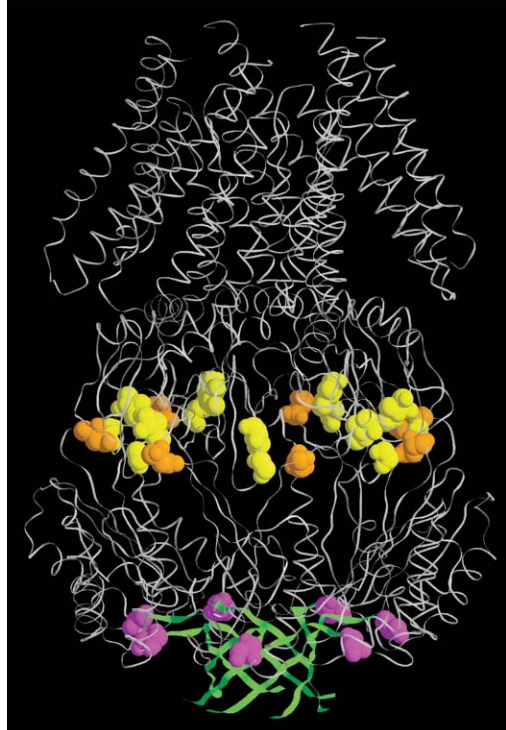

FIGURE 2 The structure of MscS showing positions of mutations within the cytoplasmic chamber that affect channel gating. Double mutations G160R and I162E (indicated in yellow) and single mutation N177C (indicated in orange) in β-domain of the chamber yielded nonfunctional channels. Truncated channels with deletions below serine 267 were not found in the membrane, suggesting that the channel assembly was impaired (Schumann *et al.*, 2004). The β-barrel structure (indicated in green) could be deleted but the truncated channel shows altered gating.

MscS gating than the large ones, suggests that the openings of the cytoplasmic chamber remain accessible to them in various conformational states of the channel (Grajkowski *et al.*, 2005).

The relevance of the C-terminal domain in the channel assembly and/or its ability to function has been tested in a series of mutants with deletions within their C-terminal domains. Deletions at the MscS C-terminal that were longer than the last 20 residues yielded proteins that did not incorporate into the membrane (Schumann *et al.*, 2004). Removal of the last 20 amino acids was tolerated; the protein was found in the membrane and it formed a functional channel. However, the properties of the truncated channel were altered: the channel activated and inactivated, but the recovery from the

inactive state was impaired. After the channel inactivated, it lost its ability to make a transition from the inactivated to the closed state, from which it could reopen again. Interestingly, addition of poly-histidine tag to the end of the truncated C-termini promoted a transition back to the closed state and the mutated channel functioned similarly to the wild-type one (Schumann *et al.*, 2004). This result is reminiscent to the finding that replacement of highly conserved cytoplasmic T1 domain in voltage K (Kv) channels by an artificial tetramerization module restored those channel properties that were missing in the channel lacking T1 (Zerangue *et al.*, 2000). The assembly of the Kv channels lacking the T1 domain was greatly improved in the presence of the artificial tetramerization domain. Since artificial heptamers are not available as yet, it is not possible to investigate if potential difficulties in assembly of the MscS with truncated C-termini are accountable for the lack of functional channels.

Except the channels with truncated C-termini, some mutants with single or double substitutions within C-terminal chamber have been tested electrophysiologically. It has been found that the double substitution of highly conserved residues in the middle β-domain G160R and I162E (marked in yellow in Fig. 2) or the single substitution N177C in the same region (marked in orange in Fig. 2) both yielded nonfunctional channels (Koprowski *et al.*, unpublished data).

V. STRUCTURAL ALTERATIONS OF THE MscS CYTOPLASMIC CHAMBER ON GATING

The data mentioned above indicate that the cytoplasmic chamber may undergo large conformational changes on transition from the closed to open state of the channel. By analogy, similar changes in the opposite direction may be expected on transition from the open to inactivated and then back to the closed conformation. In effort to predict these structural rearrangements, a series of experimental conditions was set, under which changes in the surface of the MscS cytoplasmic chamber and/or in its entire ion-conducting pathway could be expected, and resulting alterations of the channel kinetics would be observed. It is well known that large molecules (cosolvents) present in the solution surrounding the protein of interest interact with it (Timasheff, 1998, 2002). The interaction can be positive (preferential binding) or negative (preferential exclusion resulting in preferential hydration). Cosolvents that preferentially bind to proteins (urea, guanidine hydrochloride, or propyleneglycol—solubilizers) are known to solubilize and denaturate proteins and promote an expanded, unfolded state. On the other hand, cosolvents that are preferentially excluded from proteins [polyethylenoglycans (PEGs), dextrans, ficoll, and sucrose—stabilizers] fix them in the native, compact state. In an

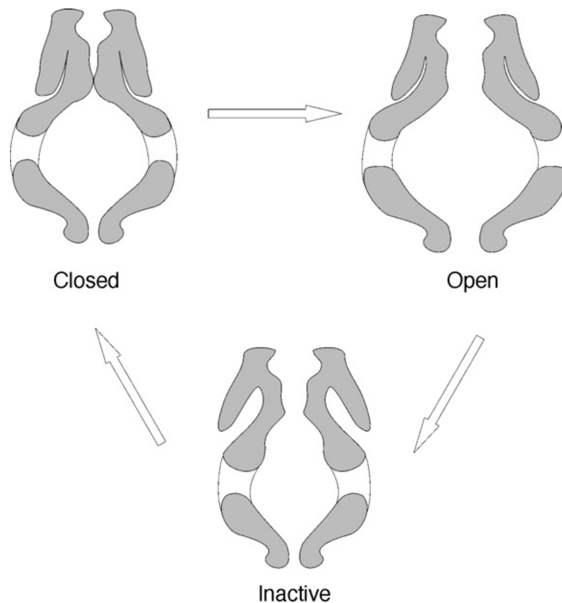

FIGURE 3 Model of MscS conformational changes on activation, inactivation, and closing based on available experimental data and existing models. On the channel activation, the pore opens and both the inner and the outer surfaces of the cytoplasmic chamber increase. The inactivation is associated with a detachment of TM3 helices from TM1 and TM2, which results in a nonconductive pore conformation. The outer surface of the cytoplasmic chamber decreases and the chamber lowers its volume. The crystal structure is interpreted here as a representation of the inactivated conformation. On transition from the inactivated to the closed state, TM3 attaches again to TM1 and TM2 and the inner surface of the cytoplasmic chamber increases. The protein is represented as a schematic cross section so that the individual subunits twisted around MscS symmetry axis are undistinguishable.

approach combining patch-clamp analysis with a use of various-size stabilizers, it has been expected that the MscS kinetic states are linked to the conformational changes of the channel in the presence of cosolvents (Grajkowski *et al.*, 2005).

It has been found that large cosolvents that cannot enter the channelwater-filled cytoplasmic chamber impair channel activation and accelerate its inactivation when applied from the cytoplasmic side but they slow down inactivation when applied from the extracellular side. It has also been found that small cosolvents that can enter the channel, cytoplasmic chamber prevents the channel from opening much stronger than the large ones, having almost no effect on the inactivation rate. On the basis of crystal structure, possible conformational changes of the channel molecule on transitions between its functional states have been suggested and they can be summarized as follows:

1. Activation is associated with an increase of the area of the channel inner surface (the chamber and the TM gate) accessible to small cosolvents, and possibly with an increase of the volume of this entire part of the channel. Large cosolvents that interacted with the outer side of the channel cytoplasmic domains also affected activation but to a lesser degree.
2. Inactivation is associated with a decrease of the external surface of the cytoplasmic chamber of the channel and probably with a decrease of its volume. The periplasm-exposed parts of the channel enlarge their surface on inactivation.
3. On closure (a transition from inactivated to closed state) the channel increases its inner surface area.

These results are in an agreement with the previous suggestion that in the closed state cytoplasmic domains are in a much more compact conformation then in the crystal structure (Miller *et al.*, 2003).

A study utilizing steered molecular dynamics simulation revealed a widening of the channel, when restrains imposed on the channel to keep it in the crystal structure conformation were abolished, and the surface tension was applied (Sotomayor and Schulten, 2004). These data suggest that the channel may become larger than revealed by the crystal structure, and since the pore radius increases in the expanded conformation it may, indeed, represent the open channel state. Figure 3 shows possible rearrangements of MscS on gating and the presented model makes use of all available experimental data and existed models.

VI. CONCLUSIONS AND PERSPECTIVES

Since the moment of crystallization of MscS, it has been noticed that the organization as well as the size of its cytoplasmic part is reminiscent of the cytoplasmic domains of eukaryotic channels including the best characterized T1 domain of the potassium channels. It is now well established that the T1 domain from potassium K_V channels is involved not only in the channel assembly but also in gating by stabilization of the closed conformation of the channel, and thereby plays a key role in the conformational alterations leading to the channel opening (Cushman *et al.*, 2000; Minor *et al.*, 2000; Jiang *et al.*, 2002). The data presented in this chapter, particularly those showing that certain mutations within the MscS cytoplasmic domains prevent the channel from opening, may in fact indicate the stabilizing role of the chamber in the closed state of the channel. It is not known at present if the MscS assembly is under control of its cytoplasmic domains, however, the channels with the truncated C-termini are not found in the membrane.

Understanding the structural changes in MscS, particularly in the region of its cytoplasmic chamber, may be of great importance in understanding

how the MS channels are integrated with cell physiology. Since MscS crystallization, it has been speculated on the possibility that the cytoplasmic chamber may be a docking site for the cytoplasmic regulatory proteins (Bass *et al.*, 2002). It is well documented that cytoplasmic domains of many eukaryotic channels of various types are sensitive to the binding of cytosolic molecules that affect channel activities (Roosild *et al.*, 2004; Pegan *et al.*, 2005; Lu *et al.*, 2006). It would be of interest to explore this possibility in MscS taking into consideration a complexity, flexibility, and a potential involvement in gating of its cytoplasmic chamber. This is a sound and not new idea, an MS channel as a signal transducer has been proposed in osmotaxis (Martinac *et al.*, 1987; Li *et al.*, 1988). Bacteria respond to the abrupt changes in osmolarity by changing its motile behavior trying to avoid high or low concentrations of solutes. One substance can be either an attractant or a repellent depending on a concentration used and, therefore, it is suggested that the system sensing osmolarity responds *de facto* to the changes in concentration of water. There are two types of *E. coli* behavior to osmotic upshifts: one utilizing the chemotactic machinery and the other for which the chemotaxis system is not required. It has been demonstrated that bacterial chemoreceptors that mediate the motile behavior change their spatial organization within the lipid bilayer in response to osmotic stress (Vaknin and Berg, 2005). The other system detecting changes in solute concentration and not dependent on chemotaxis system was not resolved as yet and the involvement of MS channels in this bacterial response was proposed (Li and Adler, 1993).

The MscS-like proteins are found in other species than bacteria, and the MscS-like protein family is much diversified (Blount *et al.*, 2005). The MscS cytoplasmic chamber is a conserved structure (more than the C-terminal domain in MscL; Fig. 2), and, therefore, it can be assumed that its role might be of crucial importance for the potential mechanosensory activity of the MscS homologues. Investigation of structural rearrangements of the cytoplasmic chamber of the bacterial channel may indeed lead to better understanding of the principles of mechanotransduction on the level of single molecule and eventually integrate the detailed conformational changes of the chamber with the cell physiology.

Acknowledgments

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