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NAG2-1352 "Mechanosensitive Ion Channels in Bacteria: Functional Domains and Mechanisms of Gating"

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Summary of Research

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Summary

The past funding period was productive for the group. The progress in the mechanosensitive channel field was critically affected in the end of 1998 by the solution of the crystal structure of the mycobacterial homolog of MscL by our colleagues from Caltech. Having the structure of TbMscL in the closed state, we developed a detailed homology model of EcoMscL, and related the structural model with the wealth of functional phenomenology available for the *E. coli* version of the channel (EcoMscL). The biophysical properties of the open MscL helped to model the open conformation and infer the pathway for the entire gating transition. The following experiments provided strong support to the atomic model of the gating process, and allowed to make further predictions. The work has advanced our understanding of tension-driven conformational transitions in membrane-embedded mechanosensory proteins, determine major energetic contributions and set the stage for further exploration of the whole family of mechanosensitive channels. The results have been published in seven experimental and theoretical papers, with three other papers currently in press or in preparation.

Molecular models of EcoMscL and the hypothesis about the conformational transition. This was specific aim #5 in the original proposal. After the publication of the crystal structure of TbMscL (Chang et al., 1998), we accomplished the modeling phase in collaboration with Drs. S. Durell and H.R. Guy (NIH) (Sukharev, 2001b).

Three-dimensional structural models of the mechanosensitive channel of large conductance, MscL, from the bacteria *M. tuberculosis* and *E. coli* were developed for closed, intermediate, and open conformations. For the transmembrane region and outer loops, the modeling began with the crystal structure of *M. tuberculosis* MscL, which is a homopentameric assembly with two transmembrane α helices, M1 and M2, per subunit (Chang et al., 1998). The first twelve N-terminal residues, not resolved in the crystal structure, were modeled as an amphipathic α helix, called S1. In the modeled closed conformations, S1's from the five subunits assemble as a bundle of parallel α helices that occludes the pore, forming the cytoplasmic gate. When membrane tension induces expansion of the transmembrane region, the tilt of the transmembrane helices is postulated to increase as they move away from the axis of the pore, while the periplasmic loops incorporate into the outer part of the pore. Substantial expansion is postulated to occur before the increased stress in linkers connecting S1 to M1 helices pulls the S1 bundle apart. During the opening transition, the S1 helices and C-terminus amphipathic α helices, S3, are postulated to dock parallel to the membrane surface on the perimeter of the complex. The proposed gating mechanism reveals critical spatial relationships between main functional components such as the expandable transmembrane barrel formed by M1 and M2 helices, the gate formed by S1 helices, and 'strings' that link S1's to M1's. These models are consistent with numerous experimental results and modeling criteria.

Experimental testing of the models. The role of N-terminal domains. The model of EcoMscL closely followed the crystal structure of TbMscL in the transmembrane region. In addition to that, positions of unresolved domains were inferred and orientations of domains possibly distorted by specific crystallization conditions were corrected. The major technique for testing the proximities of selected residues in the closed and open states was cysteine mutagenesis and disulfide cross-lining combined with the patch-clamp technique. The first evidence that the N-terminal (S1) domain and the S1-M1 linker are involved in gating was reported in (Sukharev et al., 2001a). The role of N-terminus was difficult to predict before we accomplished first rounds of modeling and attempted to relate the transition with kinetic and thermodynamic properties of the channel. It was not mentioned in the original proposal, and therefore was a new find.

The mechanosensitive channel of large conductance, MscL, is a ubiquitous membrane-embedded valve involved in turgor regulation in bacteria (Sukharev et al., 1997; Levina et al., 1999). The crystal structure of MscL from *Mycobacterium tuberculosis* (Chang et al., 1998) provides a starting point for analyzing molecular mechanisms of tension-dependent channel gating. We developed structural models in which an inner gate is formed by a bundle of five cytoplasmic N-terminal helices (S1), previously unresolved in the crystal structure. When membrane tension is applied, the transmembrane barrel expands and pulls the gate apart through the S1-M1 linker. We tested these models by substituting cysteines for residues predicted to be near each other only in either the closed or open conformation. Our results demonstrate that S1 segments form

the bundle when the channel is closed, and cross-linking between S1's prevents opening. S1's interact with M2 when the channel is open, and cross-linking of S1 to M2 impedes channel closing. The gating is affected by the length of the S1-M1 linker in a manner consistent with the model, revealing critical spatial relationships between the domains that transmit force from the lipid bilayer to the channel gate.
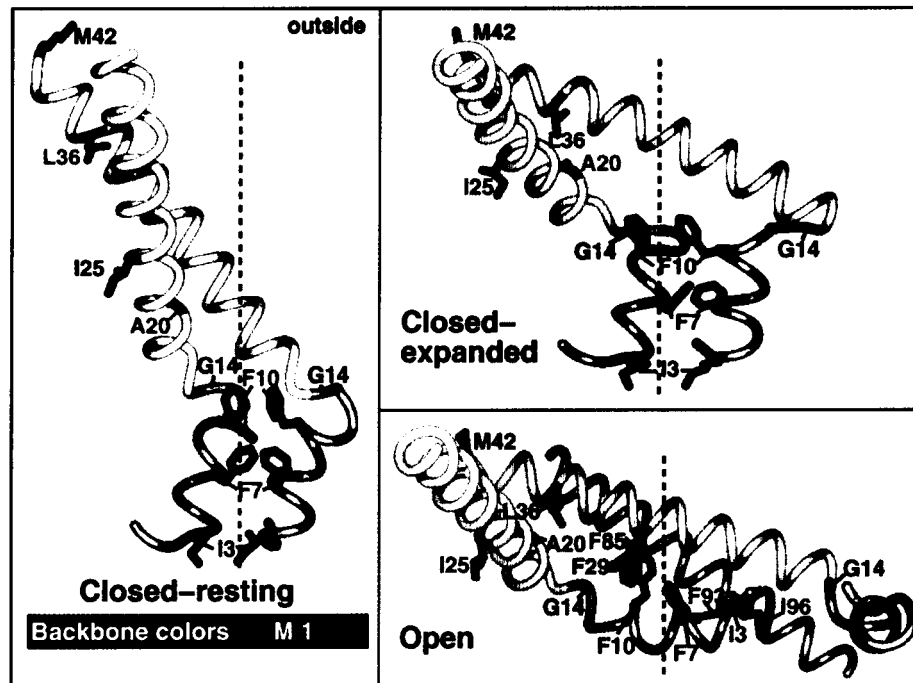

Fig. 1. The fragment of EcoMscL model depicting N-terminal S1 helices (pink), M1 helices (yellow), and the S1-M1 linkers containing the critical glycine 14 (gray). The bundle of S1 helices forms the additional cytoplasmic gate. The linkers are proposed to be force-transmitting elements between the transmembrane barrel and the cytoplasmic gate. The critical residues are shown in stick representation.

The conformation of the transmembrane barrel in the open state. We have been able to apply the same combined approach to test the highly tilted conformations of transmembrane helices forming the transmembrane barrel of the channel in the open state, and, in part support the hypothesis of a gradual iris-like expansion (Betanzos et al., 2002). This part was not foreseen in the original proposal formulated before the crystal structure was published, and therefore is new.

Under extreme hypoosmotic conditions MscL forms a large non-selective pore that protects the cell from lysis by releasing small osmolytes. Molecular models of its gating mechanisms are tested here. Disulfide cross-linking shows that M1 transmembrane α helices in resting *E. coli* MscL channels are arranged similarly to those in the crystal structure of MscL from *M. tuberculosis*. An expanded conformation was trapped in osmotically shocked cells by the specific disulfide bridging between cysteines of adjacent subunits introduced at positions 20 and 36 of the M1 helices. These bridges stabilized the open channel. Disulfide bonds engineered between the M1 and M2 helices of adjacent subunits (C32 to C81) do not prevent channel gating. These two findings support gating models in which interactions between M1 and M2 of adjacent

subunits remain relatively unaltered while their tilts simultaneously increase dramatically as tension pulls them away from the axis of the pore. The MscL barrel apparently undergoes a large concerted iris-like expansion and flattening when perturbed by membrane tension.
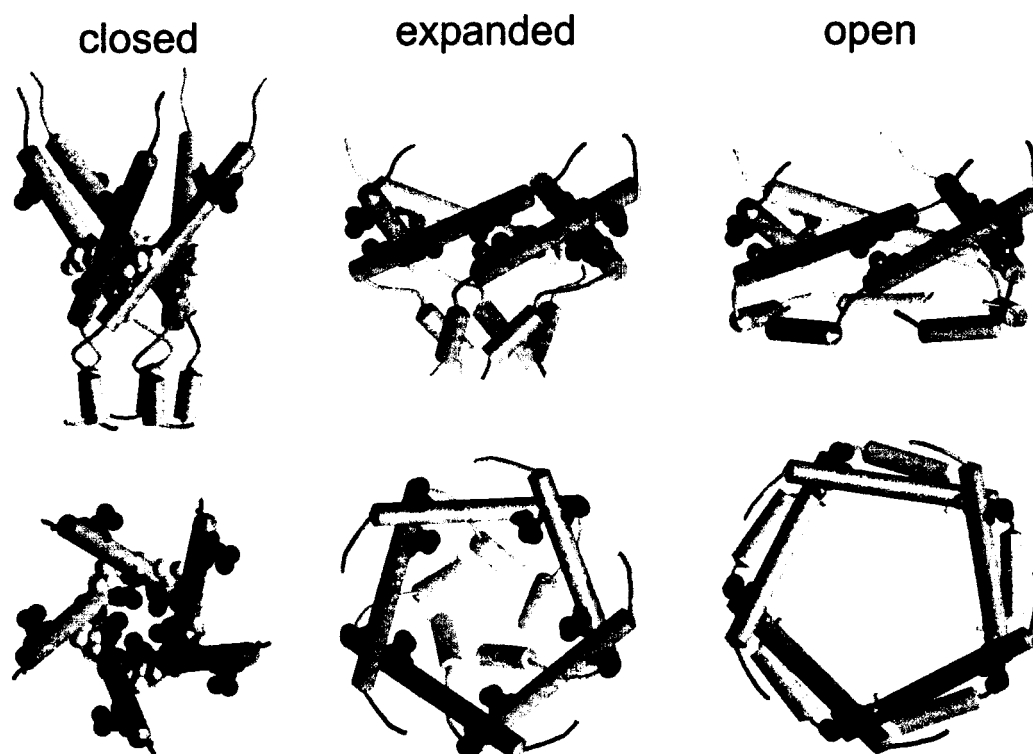

Fig. 2. The sequence of conformations illustrating the iris-like movement of the inner M1 helices one along another driven by external tension. The critical residues are shown in the VdW representation. The cross-links between cysteines grafted instead of G26(green) and I24 (yellow) confirm the closed conformation. The cross-links between A20C (orange) and L36C (purple) lock the channel in the expanded or open state.

The conformation of the cytoplasmic (S3) domains was found unusual in the crystal structure of TbMscL and was corrected in the EcoMscL homology model. In the initial model of the open state S3 domains were predicted to separate and attach to the cytoplasmic side of the barrel. There was no experimental data supporting this hypothesis. Further experimental and computational studies have shown that the separation of S3 domains in the course of opening is highly unlikely. The stable association of these domains has been demonstrated and the functional role of the bundle-like assembly was found (Anishkin, el al. 2003b):

C-terminal (S3) domains are conserved within the MscL family of bacterial mechanosensitive channels, but their function remains unclear. The X-ray structure of MscL from *Mycobacterium tuberculosis* (TbMscL) revealed cytoplasmic domains forming a pentameric bundle (Chang et al., *Science* 282:2221). The helices, however, have an unusual orientation in which hydrophobic sidechains face outside while charged residues face inside, possibly due to specific crystallization conditions. Based on the structure of pentameric cartilage protein COMP, we modeled the C-terminal region of *E. coli* MscL to better satisfy the hydrophobicity criteria, with

sidechains of conserved aliphatic residues all inside the bundle. Molecular dynamic simulations predicted higher stability for this conformation compared to one modeled after the crystal structure of TbMscL, and suggested distances for disulfide trapping experiments. The single cysteine mutants L121C, and I125C formed dimers under ambient conditions and more so in the presence of an oxidant. The double-cysteine mutants, L121C/L122C and L128C/L129C, often cross-link into tetrameric and pentameric structures, consistent with the COMP-like model. Patch-clamp examination of these double mutants under moderately oxidizing or reducing conditions indicated that the bundle cross-linking neither prevents the channel from opening nor changes thermodynamic parameters of gating. Destabilization of the bundle by replacing conservative leucines with small polar residues, or complete removal of C-terminal domain (Δ110-136 mutation), increased the occupancy of subconducting states but did not change gating parameters substantially. The Δ110-136 truncation mutant was functional in *in vivo* osmotic shock assays, however the amount of ATP released into the shock medium was considerably larger than in controls. The data strongly suggest that in contrast to previous gating models (Sukharev, 2001), S3 domains are stably associated in both closed and open conformations. The bundle-like assembly of cytoplasmic helices provides stability to the open conformation, and may function as a size-exclusion filter at the cytoplasmic entrance to the MscL pore, preventing loss of essential metabolites.
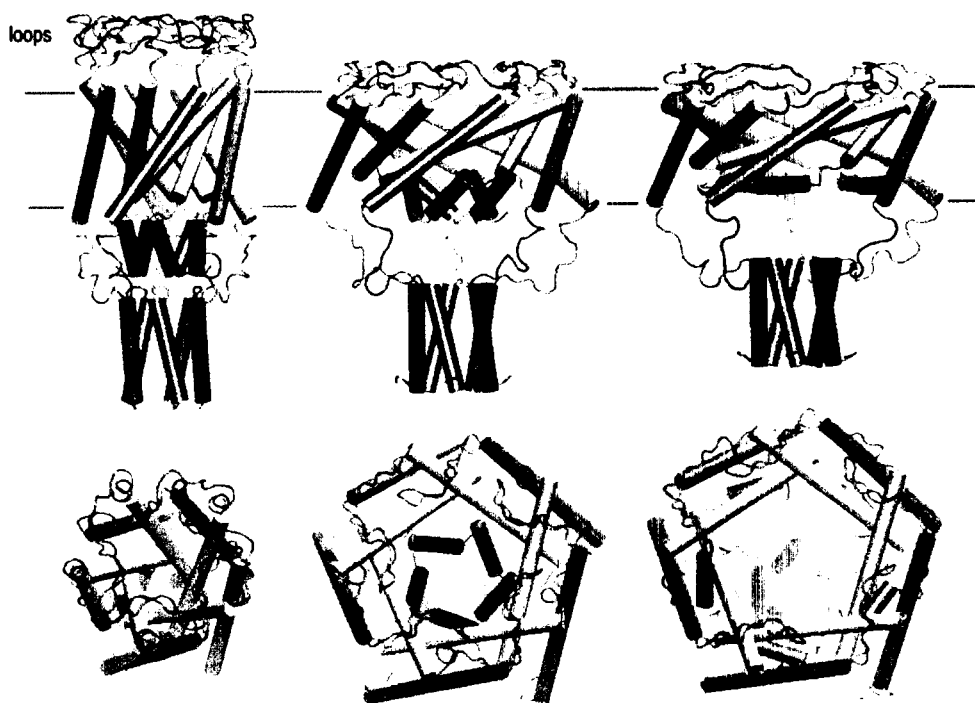

Fig. 3. Summary of the conformational transition in MscL. When the tension is applied, the transmembrane barrel (M1 (yellow) and M2 (teal) expands while cytoplasmic gate (S1, red) remains closed. Shortly after the barrel expansion, the cytoplasmic gate opens. The S3 domains are connected to M2 helices via flexible linkers and remain associated together in all conformations, forming a pre-filter at the cytoplasmic entrance to the pore.

What is the nature of subconducting states? The explanation of deviations of channel conductance form the fully open level as violations of channel cooperativity was sought. This was specific aim #4 in the original proposal. The analysis of single-channel currents revealed that there are clearly two types of subconducting states, short-lived and long-lived (Anishkin et al., 2003a). Due to the drastic difference in characteristic dwell times, they have been ascribed to the dynamics of different domains of MscL. The short-lived substates were proposed to be intermediate conformations in the path from the closed to the open states and attributed to the events of assembly-disassembly of mobile N-terminal (S1) domains (Sukharev et al., 2001a). The long-lived substates were more occupied at higher tensions (and higher voltages) and were classified as alternative open states. It was found that long-lived substates are much more pronounced in the truncated $\Delta 110$-136 mutants lacking S3 domains (Anishkin et al, 2003b). The bundle-like assembly of S3 helices apparently stabilized the fully open state and made the transitions more cooperative. Therefore, the long-lived substates were interpreted as asymmetrical conformations of the transmembrane barrel.

What is the energetic impact of critical residues in the M1 domain? This was the specific aim #2 in the original proposal. The critical residues are the pore-lining residues in the narrow part of the closed pore forming the M1 gate. The hydrophobic to hydrophilic substitutions in this region were shown to render the channel easy to open. The assessment of closed-to-open transition energies in WT EcoMscL, a 'mild' mutant V23T and two severe gain-of-function mutants V23D and G22N has been done. The values of transition energy and lateral expansion of the protein for the WT MscL have been recently revised. The expansion by 20 nm^2 is accompanied by a large energy increase of about 55 kT (32.4 kcal/mole). The hydrophilic V23T substitution shifts the activation curve slightly to the left and reduces the energy of the open state to about 40 kT. The charged substitution V23D increases the channel propensity to the low-conducting substate. It was also shown to de-stabilize the closed state by about 23 kT, and pre-expand the channel by about 5 nm^2. The data are currently in the final stage of analysis and the manuscript is in preparation.

Are hydrophobic interactions involved in setting the tension for MscL activation? Answering this question was the specific aim #1. The answer is yes. We have not pursued the experiments with chaotropic/stabilizing agents, however. The analysis of gain-of-function mutants above clearly indicates that hydrophobic interactions in the apparently dehydrated constriction of MscL stabilize the closed state. Hydration in the presence of charges (gain-of-function substitutions in the middle of the pore) clearly destabilizes the closed state. The dependence of free energy for opening on temperature is unusual: the channel is stiffer at higher temperature and easier to open when cooled down. This is consistent with the hydrophobic nature of interactions in the closed gate. The entropic contribution in the total energy of the transition may have several components (sources) and picture is rather complex. A possible contribution of lipid distortion around the flattened channel protein is currently being analyzed.

We have not analyzed **the role of periplasmic loops** experimentally (aim #3). The preliminary molecular dynamic simulations, however suggested the possible role of charges in the exapandability of loops. The charge substitution mutants have been generated, but the analysis was delayed due to a shortage of manpower and different prioritization of tasks.

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