The dynamics of protein-protein interactions between domains of MscL at the cytoplasmic-lipid interface

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Introduction

The bacterial mechanosensitive channel of large conductance, MscL, is one of the best characterized mechanosensitive channels serving as a paradigm for how proteins can sense and transduce mechanical forces. The physiological role of MscL is that of an emergency release valve that opens a large pore upon a sudden drop in the osmolarity of the environment. A crystal structure of a closed state of MscL shows it as a homopentamer, with each subunit consisting of two transmembrane domains (TM). There is consensus that the TM helices move in an iris-like manner tilting in the plane of the membrane while gating. An N-terminal amphipathic helix that lies along the cytoplasmic membrane (S1), and the portion of TM2 near the cytoplasmic interface (TM2ci), are relatively close in the crystal structure, yet predicted to be dynamic upon gating. Here we determine how these two regions interact in the channel complex, and study how these interactions change as the channel opens. We have screened 143 double-cysteine mutants of E. coli MscL for their efficiency in disulfide bridging and generated a map of protein-protein interactions between these two regions. Interesting candidates have been further studied by patch clamp and show differences in channel activity under different redox potentials; the results suggest a model for the dynamics of these two domains during MscL gating.

Upon gating, the best evidence suggests that the two transmembrane domains tilt within the membrane; as they do so, the first transmembrane TM1 twists in a cork-screw-like manner in a clockwise direction (when observed from the periplasm), and the channel then opens like the iris of an old-fashion camera. Note that in the crystal structure a vestibule of the closed pore is observed within the periplasmic side of the membrane, with the pore constriction occurring in the cytoplasmic region of the transmembrane domains. Several studies have shown that this cytoplasmic-constriction region is important for channel gating. The N-terminal helix (S1) lies along the membrane and has been shown necessary for channel gating.13,14 It appears to anchor TM1 to the cytoplasmic side of the membrane and is thought to guide its tilting within the membrane during the gating process.15,16 The region of TM2 near the cytoplasmic interface, (TM2ci; Fig. 1), has also been shown to influence the gating process. One of the residues within this subdomain, N103, has been shown to be incorporated into the membrane upon gating, presumably to accommodate for the longer tilted TM domains in the open state. Several additional residues in the TM2ci region are also crucial for this transition, thus tuning the gating of the channel.

Although the structural data from the MscL crystal structure shows that the S1 and TM2ci domains are in very close proximity (Fig. 1B), little is known about protein-protein interactions in this region and their possible influence in the gating process. This is due to the fact that the cytoplasmic interface is a difficult location to access, and that the region lacks a defined interface that can be observed in cryo-electron microscopy. In particular, the region comprising the N103 residue is difficult to access using this technique. It is known, however, that the cytoplasmic region is important for channel gating, and that therefore there must be protein-protein interactions that are not resolved in the crystal structure. We have therefore screened for protein-protein interactions in the cytoplasmic interface using a disulfide trapping approach. This technique allows for the trapping of protein-protein interactions by incorporating cysteine residues into the two interacting domains and allowing disulfide bridges to form between them. Differences in channel activity under different redox potentials were screened, and the results provide insight into the dynamics of these protein-protein interactions during channel gating.

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no manipulation of the protein and is therefore more likely to reflect real protein-protein interactions, eliminating possible artifacts occurring during protein purification. Briefly, bacteria expressing each double mutant were grown in high salt media and either diluted in media of the same osmolarity (control) or lower osmolarity (osmotic down-shock) with the oxidizing agent copper-phenanthroline. After a brief incubation the bacteria were pelleted, resuspended in sample buffer, run in an electrophoresis gel and visualized in a western blot with a MscL antibody. Interestingly, we found that interactions between S1 and TM2_\text{ci} domains are so efficient that only 1.5 μM copper-phenanthroline had to be used in order not to saturate the screen.

To compare the likelihood of interactions between cysteine substituted residues from S1 and TM2_\text{ci} domains, we measured the amount of protein existing as monomer through pentamers in western blots. We expressed the values as the percentage of multimers vs. the total protein. For the multimer value we counted only the trimers to pentamers because some single cysteine mutants in S1 can form dimers, thus leading to ambiguity. The 3D graph in Figure 2 summarizes our results. The X and Y axis are the residues from S1 (residues 2 to 12) and TM2_\text{ci} (residues 93 to 105) regions respectively, and the z axis reflects the percentage of total protein existing as multimers for each double cysteine mutant. Note that the graph shows a pattern where almost no multimer formation occurs between S1 and residues A95, M94 and F93, residues that are inside the membrane of TM2, while the strongest interactions occur between K105 to L102 and S1.

The double mutant M12C/N103C showed one of the higher values in multimer formation. These residues are predicted to interact in the closed MscL, and as can be seen in the western blot, the mutated protein exists exclusively as a pentamer after treatment with oxidizer (Fig. 3A and B). Furthermore, M12C/N103C single channel activity could only be seen after treatment with the reducing agent DTT, and the activity disappeared when oxidizer was added to the bath (Fig. 3C). These results are consistent with M12C/N103C MscL being locked in a closed conformation when disulfide bridging occurs.

On the other side of the graph, one of the peaks reflecting multimerization is I3C/I96C. These residues are not predicted to interact in the closed MscL, and as can be seen in the western blot, the mutated protein exists exclusively as a pentamer after treatment with oxidizer (Fig. 4A and B). Patch clamp experiments show that I3C/I96C single channel activity can be elicited both in the presence of reducing or oxidizing agents in the bath (Fig. 4C). Interestingly, a more detailed analysis of the amplitude of the channel in different redox conditions showed that in the presence of the oxidizer the channel is stabilized in a sub-conductive state (Fig. 4C and D).

Finally, to better understand the interactions occurring between S1 and TM2_\text{ci} domains, an additional number of double mutants were analyzed by patch clamp and the results are shown in Figure 5. Mutants I3C/K97C, I3C/N100C and E6C/K101C showed, like I3C/I96C, stabilization in a sub-conductive state.
upon oxidation, but did not lock closed. The mutants M12C/R104C, M12C/N100C, E9C/K101C and E6C/I96C, similar to M12C/N103C, locked closed under oxidation; however, more substates were observed for mutants in the middle of the two regions and sensitivity to oxidation varied among mutants.

Discussion

MscL has been shown in previous studies to be, in general, quite tolerant of cysteine mutations.9 Perhaps partially because of this finding, cysteine trapping experiments have been utilized to address proximity of regions of the protein that are thought to interact. However, in some instances, researchers have been misled by utilizing targeted mutagenesis and disulfide trapping experiments, proposing models later shown to be false. For example, S1 was thought at one time to form a helical bundle that served as a second gate.18,19 The major form of evidence for this model was targeted disulfide trapping. However, when the entire region was scanned and disulfide trapping assessed and compared,15 the results were consistent with the newly re-evaluated structure: S1 was an amphipathic helix running along the cytoplasmic membrane. There was no evidence that a S1 “second gate” was ever formed during the gating process. We have designed the experiments used in this study to avoid some of the many potential pitfalls of the disulfide trapping approach. First, we have not used a targeted approach. Instead, we have generated and screened 143 double cysteine mutants, which act as controls for each other; relative amounts of disulfide bridging can easily be assessed. Second, the amount of oxidant used was carefully titrated to yield the maximum range of disulfide bridging; none of the interactions were forced by high concentrations of oxidant. Indeed, the amounts used in this study are 100 times less than that used to study TM1/TM2 interactions, where the interactions may be more insular and transient upon gating.17 Finally, we use an in vivo approach in which the membrane proteins are not solubilized prior to being resuspended in SDS running buffer; hence disulfide bridges cannot form in mild detergents that would preserve the pentameric complex while possibly allowing more dynamic interactions not reflecting any normal physiological state. We believe that the following of these practices when utilizing a disulfide trapping approach yields a more true and interpretable set of data.

Our current model for S1 function is that it serves as a stabilizer for the twisting and turning of the pore-forming first
transmembrane domain. In addition, all current models for the structural changes that occur upon gating include a tilting of both transmembrane domains within the plane of the membrane. In addition to tilting, for TM1 several lines of evidence also suggest a clockwise “corkscrewing” of TM1 of almost 180°, as would be observed from the periplasm; these lines of evidence include EPR experiments, disulfide trapping experiments within the pore and between TM1 and TM2 upon gating, accessibility and gating influences of the sulfhydryl reagent MTSET on TM cysteine mutants, and the engineering of heavy metal binding sites within the pore region. The S1 amphipathic helix, with its two conserved phenylalanines, is joined to this dynamic TM1 by a conserved flexible glycine residue at position 14 that has been shown to have functional significance. Such a helix running along the membrane is observed in many channels including the other mechanosensitive channel in bacteria, MscS, the “slide helix” of KirBac1.1, a putative amphipathic “gate anchor” domain studied in TRPY1, and several others where they may also serve as stabilizers.

Although TM2 is not thought to “cork-screw” within the membrane, it is thought to tilt within it upon gating. Indeed, a recent study of the dynamics of the TM2 ci region has shown that channel gating can be influenced by modifications that make this region more hydrophobic. One residue, N103, was even shown to transiently insert into the membrane during the gating process in a piston-like manner. Hence, this region shows a dynamics within and along the membrane upon channel opening. The proximity of the S1 and TM2 ci regions is obvious in the closed M. tuberculosis MscL structure. An analysis of the equivalent residues to E. coli M12 and N103 is entirely consistent with our results that these residues, when mutated to cysteines, readily disulfide bridge and form a channel resistant to opening. On the other hand, several residue combinations, including the distal I3C and I96C, are not predicted to be close in the M. tuberculosis structure, do not lock the channel in the closed state when oxidized, but do stabilize substates. These data are consistent with the models for the dynamics of the S1 and TM2 ci outlined above: upon gating the S1 helix slides along the cytoplasmic membrane, where it can interact with residues further up in the membrane portion of TM2 because of the tilting of this latter domain. Thus, overall the double cysteine mutants that bridge the tension in the membrane; more specifically, changes within the lateral pressure profile. The observation that many other mechanosensors also have lower thresholds or are activated by amphiphats suggests this is a common stimulus for MS channels. Furthermore, the observation that the channel can function in a bilayer composed of phosphatidylcholine, which contains a zwitter-ionic headgroup not synthesized by E. coli, demonstrates that neither interactions with negatively charged lipids nor native lipid headgroups are required for normal MS channel activity. Both transmembrane domains tilt within the membrane, and TM1 rotates in a corkscrew fashion clockwise, as observed from the periplasm. Finally, this study has helped to define the interactions between the S1 and the TM2 ci domains; the findings are consistent with the model of S1 maintaining its association with the cytoplasmic side of the membrane and thus serving as a stabilizer of these regions upon gating that show stabilized subconducting states when oxidized.

Collectively, the data from this study, as well as many others, have led to a cohesive model of how MscL senses and responds to mechanical forces. We know, for instance, that MscL senses the tension in the membrane; more specifically, changes within the lateral pressure profile. The observation that many other mechanosensors also have lower thresholds or are activated by amphiphats suggests this is a common stimulus for MS channels.
Figure 5. Correlation between multimer peaks and channel activity of MscL double mutants under different redox potentials. In a simplified version of the 3D graph shown in Figure 2, the multimer peaks for seven additional mutants are highlighted with red (mutants that lock close upon oxidation) or green (do not lock close upon oxidation). The inserts show the corresponding position in *M. tuberculosis* model of the substituted residues in *E. coli* MscL. Just two subunits are shown for clarity; residues in green are in S1 and those in red in TM2. For each mutant the traces show single channel activity under reducing (black) or oxidizing (blue) conditions. The number under each trace is the negative pressure applied to the patch. Scale bars on the right bottom apply to all traces.

for the pore-forming TM1, and the dynamics of the TM2 sub-domain and the predicted tilting of TM2 within the membrane.

**Materials and Methods**

**Strains and cell growth.** *E. coli* strain PB104 (*ΔmscL::Cm*),[10] was used as host for the pB10 expression constructs.[14-32] PB104 was used for the in vivo cysteine trapping experiments and for the generation of spheroplasts for electrophysiological analysis. Cultures were routinely grown in Lennox Broth (LB) media (Fisher Scientific) plus ampicillin (100 mg/ml) in a shaker-incubator at 37°C and rotated at 250 cpm. Expression was induced by addition of 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) (Anatrace).

**Generations of mutants.** Single mutants were generated using the mega primer technique as described.[7] The double mutants
were generated by digesting with restriction enzymes NsiI and BamHI, which separate the DNA encoding TM1 from that encoding TM2. The two fragments encoding the cysteine mutations were then ligated to generate the insert encoding the double cysteine mutants.

In vivo disulfide trapping. Overnight cultures were diluted 1:100 and grown 1 h at 37°C in LB + ampicillin (approximately 410 μM). LB with 1 M NaCl was then added for a final concentration of 0.5 M. Cultures were then induced with 1 mM IPTG for 1 h when an OD 600 of 0.2 was reached. Cultures were either Mock shocked (0.5 M NaCl LB) or shocked (water with 1.5 μM copper-phenanthroline) at a 1:20 dilution for 15 min at 37°C. Samples were pelleted at 4,000 g for 20 min and immediately resuspended in non-reducing sample buffer, adjusted for final OD, and run on a Criterion 4–20% gel (Bio-Rad) for western blot analysis.¹⁷ Note that in less than 5 min the samples are collected and loaded on the gel. Gels were run at 200 V for 55 min and transferred to PVDF Immobilon-P (Millipore) in Tris-Glycine, 15% methanol and 0.01% SDS for 70 min at 110 V. After blocking, the primary antibody, a nitr-MscL was diluted to 1:15,000 and incubated at room temperature for 1 h. The blot was then washed and developed with Millipore HRP substrate for 5 min and exposed to film.

Disulfide trapping quantification. The density of the bands was measured with Scion Image software (Scion Corporation). The bands in each lane were measured as two sets, monomer and dimer, and trimer to pentamers (multimers). The values are expressed as the percentage of total protein existing as multimers.

Electrophysiology. E. coli giant sporekers were generated and used in patch-clamp experiments as described previously.³³ Excised, inside-out patches were examined at room temperature under symmetrical conditions using a buffer containing 200 mM KCl, 90 mM MgCl₂, 10 mM CaCl₂, and 5 mM HEPES pH 6 (Sigma). To study redox effects in channel activity H₂O₂ 1–3% v/v or dithiothreitol (DTT) 1–10 mM (Sigma) were added to the bath. A previous study has demonstrated that this treatment has no detrimental effect on channel function.³³ Recordings were performed at -20 mV (positive pipette). Data were acquired at a sampling rate of 20 kHz with a 5-kHz filter using an AxoPatch 200B amplifier in conjunction with Axoscope software (Axon Instruments). A piezoelectric pressure transducer (World Precision Instruments) was used to monitor the pressure throughout the experiments. Data were analyzed using Clampfit9 from pClamp9 software (Axon Instruments).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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