

## The oligomeric state of the truncated mechanosensitive channel of large conductance shows no variance *in vivo*

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**Abstract:** The mechanosensitive channel of large conductance (MscL) from *E. coli* serves as an emergency release valve allowing the cell to survive acute osmotic downshock. It is one of the best studied mechanosensitive channels and serves as a paradigm for how a protein can sense and respond to membrane tension. Two MscL crystal structures of the orthologs *M. tuberculosis* and *S. aureus* have been solved showing pentameric and tetrameric structures, respectively. Several studies followed to understand whether the discrepancy in their stoichiometry was a species difference or a consequence of the protein manipulation for crystallization. Two independent studies now agree that the full-length *S. aureus* MscL is actually a pentamer, not tetramer. While detergents appear to play a role in modifying the oligomeric state of the protein, a cytoplasmic helical bundle has also been implicated. Here, we evaluate the role of the C-terminal region of *S. aureus* MscL in the oligomerization of the channel in native membranes by using an *in vivo* disulfide-trapping technique. We find that the oligomeric state of *S. aureus* MscLs with different C-terminal truncations, including the one used to obtain the tetrameric *S. aureus* MscL crystal structure, are pentamers *in vivo*. Thus, the C-terminal domain of the *S. aureus* protein only plays a critical role in the oligomeric state of the SaMscL protein when it is solubilized in detergent.

**Keywords:** mechanosensitive channel; oligomerization; detergent solubilization; stoichiometry; disulfide trapping

### Introduction

The *E. coli mscL* was the first gene definitively shown to encode a mechanosensitive channel activity.<sup>1</sup> The encoded mechanosensitive channel of large conductance (MscL) protein (EcoMscL) is one of the best studied

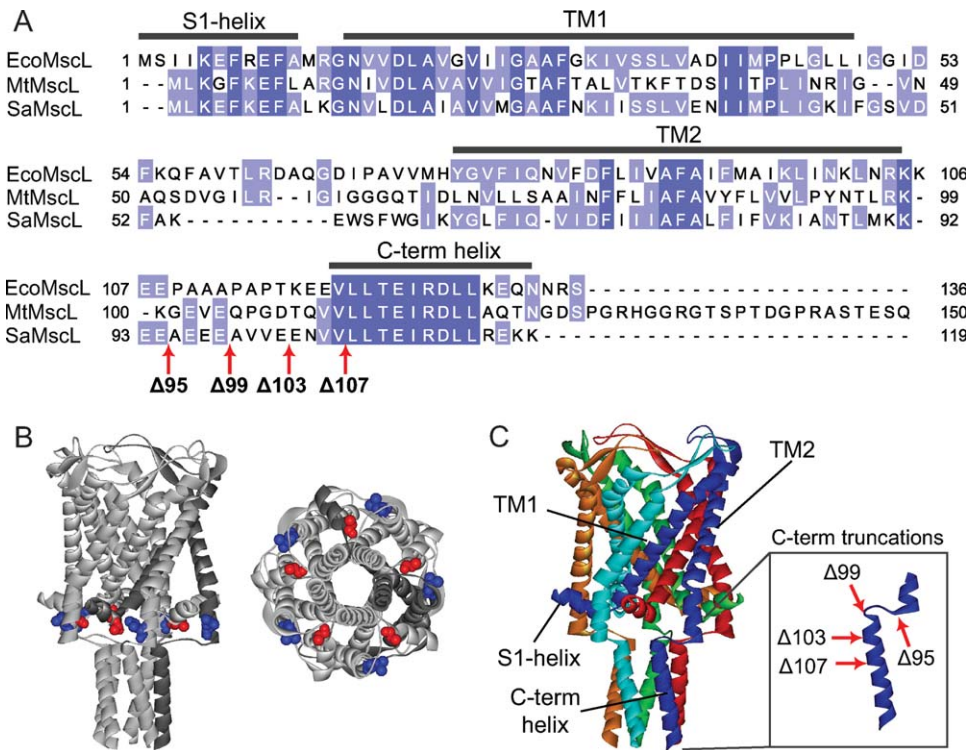
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*Abbreviations:* C8E5, pentaethylene glycol monoethyl ether; LDAO, *n*-dodecyl-*N,N*-dimethylamine-*N*-oxide; DDM, *n*-Dodecyl  $\beta$ -D-maltoside; OG, *n*-Octyl- $\beta$ -D-glucopyranoside; EcoMscL, *E. coli* MscL; MtMscL, *M. tuberculosis* MscL; SaMscL, *S. aureus* MscL; SDS, sodium dodecyl sulphate; DTT, dithiothreitol; OCAM, oligomer characterization by addition of mass.

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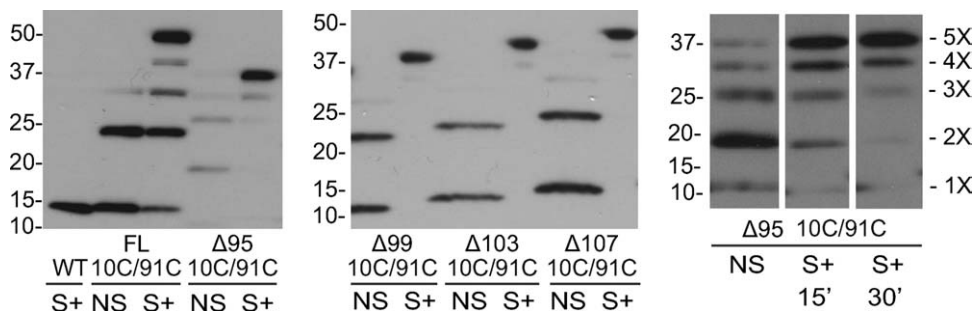
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**Figure 1.** Alignment of MscL homologs and protein modifications. **A:** Sequence alignment of three MscL homologs from *E. coli* (EcoMscL), *M. tuberculosis* (MtMscL), and *S. aureus* (SaMscL) showing the regions corresponding to the different protein domains. The degree of conservation is color coded with dark blue residues indicating identity and light blue similarity. The red arrows at the C-terminal end of SaMscL sequence indicate the sites where the stop codons were added to generate the different C-terminal truncated constructs. **B:** Localization of residues A10 (red) and L97 (blue) in *M. tuberculosis* MscL, corresponding to L10 and M91 in SaMscL. These residues were substituted to cysteines for the *in vivo* disulfide trapping experiments. A lateral view (left) and bottom view (right) are shown. **C:** Crystal structure of *M. tuberculosis* MscL, showing a pentameric stoichiometry. The inset shows a detail of the C-terminal domain and the location of the C-terminal truncations.

mechanosensitive channels, serving as a paradigm for how a protein can sense membrane tension.<sup>2</sup> A crystal structure from *M. tuberculosis* MscL (MtMscL) was first obtained by Douglas Rees' group in 1998, depicting what appeared to be a closed state of the channel.<sup>3,4</sup> It was a homopentamer with each subunit containing two transmembrane domains and a cytoplasmic C-terminal  $\alpha$ -helical bundle. The same group recently obtained a second crystal structure from a C-terminal truncated *S. aureus* MscL (SaMscL) in what they speculated to be an expanded intermediate state; surprisingly, this was a tetramer.<sup>5</sup> A different oligomeric state was unexpected given that the two orthologs are highly conserved [Fig. 1(A)], as is the entire MscL family.<sup>6–8</sup> To further investigate the discrepancy between the stoichiometry of these two channels, several studies have been performed. In one study, we developed an *in vivo* disulfide-trapping assay to determine the state of the *S. aureus* channel when in its native membrane environment; it was unambiguously a pentamer, demonstrating that the crystal did not reflect a native state of the protein.<sup>9</sup> Thus, two possibilities existed: either the truncation of the C-terminal bundle altered channel stoichiometry to

tetramer, or the detergent solubilization did. Using crosslinking, sedimentation equilibrium centrifugations and light scattering, we concluded that on solubilization with the detergent *n*-dodecyl-*N,N*-dimethylamine-*N*-oxide (LDAO), which was used to obtain the crystal structure, the channel reversibly reorganizes as a tetramer; in contrast, TritonX-100 and pentaethylene glycol monoethyl ether (C<sub>8</sub>E<sub>5</sub>) detergents did not alter stoichiometry.<sup>9</sup> However, the influence of protein truncation was not assessed. Indeed, one previous study of *in vitro* translated and *n*-Octyl-Beta-D-Glucopyranoside (OG) solubilized EcoMscL implied that the C-terminal end of the protein played a critical role in assembly.<sup>10</sup> Recently, using a new technique coined oligomer characterization by addition of mass (OCAM), the Rees' group confirmed that both MtMscL, and full length (FL) SaMscL are pentamers when solubilized in *n*-Dodecyl  $\beta$ -D-maltoside (DDM); but they also showed that a SaMscL with the C-terminal deletion, as used in the crystallographic study, was heterogeneous in its oligomeric state, existing as both pentamers and tetramers. Thus, it appeared that the C-terminal helical bundle does influence the oligomeric state of the protein. However, because both of these



**Figure 2.** Disulfide trapping experiments show that C-terminally truncated SaMscL protein exists as a pentamer *in vivo*. Western blot analysis of WT SaMscL, double cysteine mutant 10C/91C SaMscL FL and with  $\Delta 95$  truncation (left panel). All samples were either nonshocked (NS) or shocked (S+) in the presence of the oxidizing agent copper phenanthroline. When shocked in the presence of copper phenanthroline the majority of 10C/91C exists as a pentamer, independent of the length of the C-terminal truncation (left and central panels). A ladder of monomer through pentamer is already present in the nonshock sample; pentamer becomes the major band when osmotically downshocked in the presence of copper phenanthroline; this occurs in a time-dependent manner (right panel). Note that no detergent solubilization before resuspension in Laemmli sample buffer is performed in these experiments.

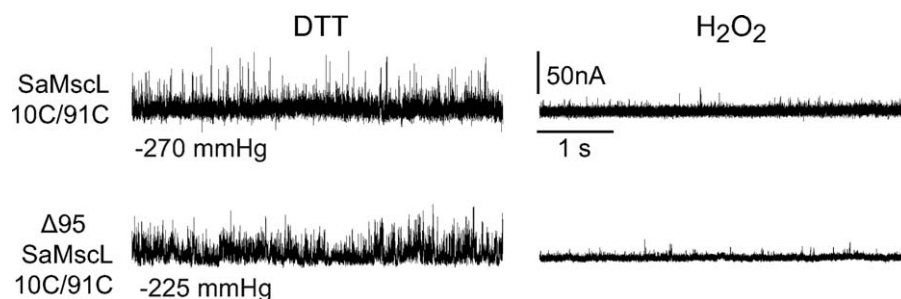
studies used detergent-solubilized proteins, it was unclear if the C-terminal region of the protein played a role in MscL stoichiometry *in vivo*. Here, we utilize our *in vivo* disulfide-trapping assay to address this issue.

To evaluate the role of the c-terminal region of SaMscL in its oligomeric state, we have generated multiple C-terminal truncated constructs of different lengths that have two cysteine mutations (L10C/M91C; Fig. 1). As can be seen in Figure 1(B), these two sites are predicted to be in close proximity in the closed state<sup>3</sup> and have been shown previously to generate the most efficient *in vivo* crosslinking.<sup>9</sup> We investigated the FL channel as well as four different C-terminal deletions:  $\Delta 95$  (which was the truncation crystallized as a tetramer),  $\Delta 99$ ,  $\Delta 103$ , and  $\Delta 107$ . Two of these truncations,  $\Delta 95$  and  $\Delta 99$ , are located in the linker between TM2 and the cytoplasmic helix and the other two are in the cytoplasmic helix itself [Fig. 1(A,C)]. As previously described, bacteria expressing the different constructs were given an osmotic shock in the presence of copper phenanthroline; this removes cellular reducing agents such as glutathione while adding an oxidant. The treated cells were pelleted, resuspended in sodium dodecyl sulphate (SDS) loading buffer, and the MscL protein visualized by SDS polyacrylamide gel electrophoresis (PAGE) and western blot. Note that the protein is trapped in its native membrane environment because no solubilization or protein handling is involved before SDS PAGE. As shown in Figure 2 (left and center panels), SaMscL exists almost exclusively as a pentamer regardless of the length of the C-terminal truncation. Although a ladder of monomer through pentamer can be observed for 10C/91C  $\Delta 95$  SaMscL in the no-shock/nontreated protein, in the presence of an oxidizer the pentamer becomes the prevalent band in a time dependant manner (Fig. 2, right panel). Note that for the SaMscL single

cysteine mutants under the same treatment, no other species than monomer was observed for M91C but a small proportion of L10C existed as dimers (Supplemental information). Dimerization of L10C was expected as the equivalent mutant from EcoMscL, M12C, also form dimers under similar treatment.<sup>11</sup> Finally, we show by patch clamp of native membranes that both FL and  $\Delta 95$  truncated 10C/91C SaMscL respond similarly to changes in the redox potential of the bath (Fig. 3). In the presence of the reducing agent dithiothreitol (DTT), both channels are functional and activity can be observed in response to negative pressures applied to the membrane patch (left traces). After perfusion with a 3% peroxide solution, channel activity could no longer be elicited even under high negative pressures (right traces). These results are consistent with the channels being locked closed after disulfide trapping, suggesting that in native membranes the FL and truncated channels are structural and functionally similar. Hence, *in vivo* the C-terminal helical bundle plays no detectable role in proper pentameric assembly.

Many previous studies have concluded that MtMscL and EcoMscL are also pentamers. A study using crosslinking, sedimentation equilibrium, and size-exclusion chromatography showed EcoMscL as a pentamer;<sup>12</sup> another study using crosslinking and electron microscopy reached the same conclusion.<sup>10</sup> The crystal structure of MtMscL as well as the recent OCAM analysis also showed a pentameric structure for this ortholog.<sup>13</sup> Finally, when studied by using our *in vivo* disulfide trapping in native membranes, all three MscL homologs have shown pentameric stoichiometry (Supplementary Information). Hence, there appears to be no variability in the oligomeric state of these MscL orthologs.

Collectively, the data suggest that the C-terminal domain of the SaMscL channel only plays a



**Figure 3.** The FL and truncated SaMscL 10C/91C channels are functionally similar. The electrophysiological data shows single-channel activities of both SaMscL 10C/91C FL and the truncated construct  $\Delta 95$  SaMscL 10C/91C. Channel openings are seen as upward deflections. Both channels respond equally to changes in the redox potential of the bath. In the presence of the reducing agent DTT, the channels are functional and activity can be observed at the indicated pressures (left traces), but after perfusing a peroxide solution in the bath, the channel activity is lost (right traces).

critical role in the oligomeric state of the protein when it is solubilized in detergent. Different detergents seem to have different abilities to alter MscL oligomeric structure. It appears that LDAO is the harshest detergent in this sense, leading to oligomeric rearrangement of the full-length SaMscL protein.<sup>9</sup> DDM and perhaps other detergents can lead to rearrangements of only the truncated, not FL, SaMscL.<sup>13</sup> However, there also appear to be species differences; from the OCAM study, the FL EcoMscL appears to form hexamers as well as pentamers when solubilized in DDM. In addition, the same study demonstrated that truncation of the MtMscL did not destabilize the pentameric organization when solubilized in DDM.<sup>13</sup> Hence, detergents alone can cause specific protein complexes to rearrange their oligomeric state while others appear stable. As detergent solubilization is a critical and necessary step to obtain membrane proteins for crystallization and other biophysical and structural studies, where possible, a complementary set of data assessing the oligomeric state *in vivo* seems advisable.

## Methods

### Molecular biology

FL *S. aureus* MscL and the FL L10C/M91C *S. aureus* MscL mutant were cloned into pET21a vector, which has a His-tag at the c-terminal end. Truncated *S. aureus* MscL mutants were produced by polymerase chain reaction (PCR) by adding stop codons at residues A96 ( $\Delta 95$ ), A100 ( $\Delta 99$ ), E104 ( $\Delta 103$ ), and L108 ( $\Delta 107$ ). Constructs were cloned into pet15b vector at NdeI/BamHI and transformed into PB116s.<sup>14</sup> All constructs from the pet15b vector have a his-tag at the N-terminal.

### In vivo disulfide trapping

Overnight cultures were diluted 1:100 and grown 1 h at 37°C in Luria-Bertani (LB) media (~410 mOsmolar). LB with 1M NaCl was then added for a final concentration of 0.5M. Cultures were then induced

with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 1 h when an optical density (OD) 600 of 0.2 was reached. Cultures were either Mock shocked (0.5M NaCl LB) or shocked (water with 1.5  $\mu$ M copper phenanthroline) at a 1:20 dilution for 15 min at 37°C. Samples were spun down at 4000g for 20 min and immediately resuspended in nonreducing sample buffer, adjusted for final OD, and run on a 4–20% gel (Bio-Rad) for western blot analysis.<sup>14</sup> Note that in less than 5 min, the samples are collected and loaded on the gel. The primary antibody anti-Penta His (Qiagen) was used at 1:4000 and the secondary Goat anti-Mouse horseradish peroxidase (HRP) (Bio-Rad) at 1:100,000. Blots were developed using HRP substrate (Millipore) and exposed to film.

### Electrophysiology

*E. coli* giant spheroplasts were generated and used in patch-clamp experiments as described previously.<sup>15</sup> Excised, inside-out patches were examined at room temperature under symmetrical conditions using a buffer containing 200 mM KCl, 90 mM MgCl<sub>2</sub>, 10 mM CaCl<sub>2</sub>, and 5 mM HEPES pH 6 (Sigma, St. Louis, MO). To study redox effects in channel activity H<sub>2</sub>O<sub>2</sub> 1–3% v/v or DTT 1–10 mM (Sigma, St. Louis, MO) were added to the bath. A previous study has demonstrated that this treatment has no detrimental effect on channel function; in fact, one cysteine mutant was shown to increase activity.<sup>16</sup> 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, Union City, CA). A piezoelectric pressure transducer (World Precision Instruments, Sarasota, FL) was used to monitor the pressure throughout the experiments. Data analysis was performed using Clampfit9 from Pclamp9 (Axon Instruments).

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