Chapter 6

On the carboxyl-terminus of MscL

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The mechanosensitive channel of large conductance, MscL, is one of the best-studied models for mechanosensation. Although a large body of experimental data is available, the most conserved domain of MscL, the cytoplasmic α-helical bundle (CP), remains enigmatic with respect to its role in channel functioning. Here we address exclusively the cytoplasmic bundle and its role on oligomerization/complex assembly and channel gating. Our results further define the role of the carboxyl-terminus as a stably associated α-helical bundle, maintaining the closed conformation of the channel.
Introduction

The **Mechanosensitive channel of Large conductance** (MscL) is a homopentameric membrane protein. MscL senses tension in the membrane and opens a large non-selective pore (127). So far, the transmembrane segments (TM1 and TM2) are thought to determine the function of the protein (60, 101, 145). They are connected by a periplasmic loop. Both, the C- and the N-terminus of the protein are located at the cytoplasmic side of the membrane. Within the group of identified mscL gene products the sequence of the carboxy-terminal residues constitutes the most conserved region of MscL (**Figure 1**).

**Figure 1 | Sequence alignment of MscL from several bacterial hosts shows conservation of the gate region (orange) and the carboxyl-terminus (green).** The conserved hydrophobic C-terminal motif has high sequence identity with the cartilage oligomeric matrix protein (COMP) and contains leucines arranged like in a leucine zipper motif. Thereby, the C-terminus was thought to be involved in MscL complex oligomerization. The fivefold symmetry of subunits forms a cytoplasmic bundle (CP) in the crystal structure (27, 123). Within the bundle hydrophobic residues - in each of the subunits - face another in close proximity enclosing a constraint lumen. This part of MscL shares sequence and structural identity with the
cartilage oligomeric matrix protein (COMP) (4) and therein contains a LxxxxxxxL sequence, the basic module of the leucine zipper. This supports a possible role of the CP in stabilization of the oligomeric complex or oligomerization of the protein itself. However, removal of the entire CP genetically (Eco-MscL Δ 110-136 (21)) or by enzymatic digestion (1) has shown that the protein still functions with wild-type like tension sensitivity. Together with the fact that mutations in the CP are without any effect (93), the C-terminal part of MscL has received little attention in functional studies. Even today after 20 years of research, the role of the C-terminus in function and biogenesis of MscL remains enigmatic.

Sukharev and co-workers (4) have noted that the original crystal structure of *Mycobacterium tuberculosis* MscL (Tb-MscL (27) PDB accession code 1MSL) had hydrophobic side-chains at the C-terminus exposed to the surrounding environment, which is energetically not favorable. A comparison with the crystal structure of COMP (PDB accession code 1VDF (89)), which hosts the identical conserved motif (LxEIRDLL), showed that here the hydrophobic side chains do not face outwards, but towards the neighboring subunits, such that they are buried from the aqueous environment. Additional crosslinking experiments provided evidence that the conformation depicted in the Tb-MscL crystal structure was likely to be an artifact of crystallization and supported the bundle to be arranged in a COMP-like fashion. Today, a revised version of the crystal structure is available (PDB accession code 2OAR (123)), showing a conformation like suggested by the aforementioned experiments of Sukharev and colleagues (4) (Figure 2). Current models predict different conformations for the CP upon gating of the channel. In one model the bundle is stably associated throughout the course of gating (4), whereas in the opposing model, based on electron micrographs, the α-helical bundle dissociates (147). The stable α-helical bundle model has received experimental support as MscL
remained active without significant changes in gating behavior upon crosslinking of the C-terminal region (4). Shortening the cytoplasmic loop, and thus reducing the connection between TM2 and the cytoplasmic bundle, decreased the maximum channel conductance, i.e. the channel became more tight (142), which is consistent with a stable conformation. These studies are opposed by electron microscopic work on the severe gain-of-function (GOF) mutant G22N, which is constitutively conducting without application of any additional stimulus (147). While in these experiments the closed state represented by wild-type MscL showed a central protrusion on electron micrographs, it could not be observed for the conducting channel, i.e. G22N. This was interpreted as dissociation of the C-terminus upon gating. Control experiments with truncated MscL (deletion of residues 110-136, i.e. the α-helical bundle) did not show any protrusion in electron micrographs. These data support the idea of dissociating C-terminal helices, likely moving towards the membrane, to

Figure 2 | The crystal structure of MscL. New insights from homologous motifs found in COMP (PDB accession code 1VDF (89)). The original crystal structure (1MSL (27)) was refined in 2007 (2OAR (123)) and the C-terminal structure is now consistent with the experimental data (4).
provide an energetically favorable conformation for the hydrophobic residues (147). This could serve an additional means to maintain the protein in the membrane at critical tension in the bilayer (147). Overall, the available studies denote at least three different functions for CP:

(I) a size-exclusion filter (4),
(II) a spring-like mechanism that keeps the pentameric assembly of MscL intact during the enormous expansion required for gating (4) or
(III) an associating and dissociating membrane anchor (147).

To date, undisputable evidence for any of these scenarios is lacking. Here, we aimed at generating heteropentamers of WT and Δ CP (i.e. Δ 110-136 MscL) subunits of MscL by expressing the two genes in our duet expression system (Chapter 2). Surprisingly, our results show that although the truncation yields functional complexes, co-expression of full-length and truncated MscL (Δ 110-136) does not yield intermediate heteropentameric complexes. The C-terminus, thus, has influence on the channel biogenesis or oligomer assembly/stability, but not on the tension sensitivity or conductance.

Results

Duet expression of WT and Δ 110-136 MscL - limits of MscL heterooligomerization?
On the basis of prior work (Chapter 2), we aimed for the engineering of MscL into heterooligomers having one to five full-length subunits mixed with a genetically truncated Δ 110-136 mutant. These complexes would serve a template to address questions on the stability of the α-helical bundle (Figure 3).
Heteropentameric complexes of MscL generated from WT and the C-terminal truncation mutant Δ110-136. Heteropentamers of this kind would yield insights in the role of CP in channel function and stability.

Based on our findings that all pentameric assemblies can be formed upon co-expression of two full-length, but different MscL variants (see Chapter 2), we expected random complex assembly to occur for truncated and full-length subunits. We thus co-expressed the gene encoding for WT-His and the one for Δ110-136-StrepII MscL from p2BAD. Hereby, the truncation gene was placed in MCSI and the full-length variant in MCSII. However, the selected co-expression strategy yielded mainly homopentamers (A and B) and only marginal amounts of heterooligomeric assemblies (C).

Figure 3 | Examples of heteropentameric complexes of MscL generated from WT and the C-terminal truncation mutant Δ110-136. Heteropentamers of this kind would yield insights in the role of CP in channel function and stability.

Figure 4 | Duet expression of WT-His and Δ110-136-StrepII MscL yields mainly homopentamers (A and B) and only marginal amounts of heterooligomeric assemblies (C). A Homopentameric WT-His MscL can be purified in relatively high quantities from Ni-NTA resin (~36 % of overall protein on the gel, based on 2D-densitometry on gel duplicates). B Flow-through and wash fractions of Ni-NTA sample of panel A contain high quantities of Δ110-136-StrepII MscL (~63 %), which could be recovered by StrepTactin affinity chromatography. C Sample passed through both affinity resins consecutively yielded minimal quantities (~1 %) of heteropentameric MscL.
unlike observed in Chapter 2, these two MscL variants did not form heteropentamers. Performing a two-step affinity chromatography yielded mostly WT (36 %) and Δ110-136 (63 %) homopentameric assemblies (Figure 4).

The fraction of heteropentamers was about 1 %, which prohibited detailed research on the role of the C-terminal a-helical bundle. Functional assays show the homopentameric constructs (both WT and Δ110-136) to be functional (Figures 5 and 6). The question now is: what drives the subunits into two discrete homopentameric assemblies? Within the CP, hydrophobic residues with fivefold symmetry of the subunits constitute a narrow confinement. The subunits shield each other’s hydrophobic surface from liquid exposure. As we have seen in the hydrophobic gating studies reviewed in Chapter 3 and described in Chapter 4, tightly packed side chains can be stabilized in their conformation by hydrophobic effects. If those residues are not shielding each other like in a natural homopentamer of MscL, the energetically unfavorable conformation might prevent heterooligomerization of full-length and Δ110-136 MscL and serve a rationale for our results at duet expression.

Functional comparison of full-length and Δ110-136 MscL reveals no significant differences at the ensemble level

To characterize the role of CP in MscL function, we expressed and isolated the homopentameric double mutant G22C Δ110-136. This variant of MscL can be triggered to open in the absence of tension by attaching charged moieties, i.e. MTSET, in the pore lining. Thereby, we are able to investigate whether or not the CP alters or blocks the flux of small molecules with techniques other than patch clamp. We first compared the activity of the truncated variant to full-length G22C MscL by fluorescence dequenching assay as described previously (73, Chapter 4). For that, the two MscL
variants were reconstituted at equal quantities into azolectin liposomes filled with the self-quenching dye calcein. The release of calcein can be monitored over time as an increase in fluorescence. After in situ activation of MscL with MTSET, the channel opens and calcein is released resulting in dequenching of the fluorophore. The data indicate some differences in the kinetics of the calcein efflux, and MTSET-labeled G22C Δ110-136 being somewhat more active than MTSET-labeled G22C (Figure 5).

Figure 5 | The absence of the cytoplasmic domain affects the kinetics of MscL in proteoliposomes. Full length and truncated variants of MscL were assayed by fluorescence dequenching. The net percent release of liposomal content was calculated from the increase in fluorescence. The non-specific release from liposomes with non-activated MscL was subtracted. The first-order rate constants (k) for calcein efflux through G22C (k=1.43 min⁻¹) and G22C Δ110-136 (k=2.50 min⁻¹) were obtained from fitting of the data.

Although the first-order rate constants (G22C k=1.43 min⁻¹; G22C Δ110-136 k=2.50 min⁻¹) differ a factor of almost two, we cannot exclude that these differences are due variations in the sample preparation and analysis (protein quantification, precipitation, in-gel densitometry, reconstitution efficiency etc.).
The open state of MscL is stabilized in the absence of CP

Patch clamp electrophysiology sheds light on the gating kinetics at the level of a single channel molecule. Thereby, ensemble effects, like averaging, are eliminated.

Although the truncated mutant of MscL was previously reported to behave WT-like with respect to gating parameters, i.e. tension sensitivity and conductance (1, 4, 21), our single-channel recordings of WT and Δ110-136 MscL revealed substantial differences in channel dwell time (Figure 6).

![Figure 6](image)

**Figure 6** | Single channel traces reveal prolonged openings in the absence of the carboxyl-terminal a-helical bundle (CP). A WT MscL typically remains open for up to a few hundred milliseconds (top) while Δ110-136 shows prolonged openings in the second time range (bottom). Both traces were recorded at comparable negative pressure applied to the patch, *i.e.* 105.5 mmHg for WT and 102.0 mmHg for Δ110-136.

While WT MscL usually gates rapidly between the closed and open state, the truncation mutant remains open for significantly longer periods. This is also reflected in the probability density of the open state, which increases drastically for Δ110-136. While WT MscL usually populates the fully open
state at a probability density of less than 0.05, the absence of the carboxyl-terminal α-helical bundle increases the probability density to > 0.2. This means, the channels open state is stabilized in the absence of CP, which provides a rationale for the improved kinetics observed in the calcein dequenching assay. In accordance with previous observations (1, 4, 21), other gating parameters like the conductance of substates or the fully open state remain unaltered. Thereby, we conclude, that the C-terminus remains stably associated during gating and stabilizes the closed state of the channel, while upon gating it serves a spring, forcing the channel to go back to the closed conformation.

**Discussion**

We analyzed the role of the C-terminus of MscL on oligomerization and the gating parameters of the channel. Duet expression of full-length and a CP-less mutant yielded only marginal amounts of heteropentamers, while the majority of the assemblies were homopentameric. A possible role of the CP in active sorting of the subunits by a hitherto-unknown cellular machinery remains a possibility but more likely the hydrophobic side chains prevent an energetically unfavorable conformation to form.

The homopentamers of WT and Δ110-136 MscL were analyzed on the ensemble and single molecule level to obtain insights in their gating behavior. The ensemble assay suggests an improved gating as the rate of release of calein was increased in the CP-less mutant. Single molecule studies support this contention. Our results show that MscL’s closed state is significantly more stable in the full length protein, whereas truncated MscL remains open with increased dwell-times and changes in the probability density of especially the closed and fully open states. However, gating parameters, such as the full open conductance or substates remain
identical. This excludes a possible function as a size-exclusion filter or pore plug, whose motion would block or open the conducting pathway and thereby define gating and substates. Nevertheless, the strong stabilizing effects of especially the closed state indicate that the α-helical bundle remains intact throughout the course of gating (4, 142). We therefore propose a stable backbone function for the cytoplasmic bundle, maintained by hydrophobic effects and possibly supported by salt-bridges between aspartate and arginine of neighboring subunits, both present in the highly conserved LxEIRDLL motif. This stable bundle conformation observed here and by others (4, 86, 142) is contradictory to the studies of Yoshimura et al. (147) where dissociation of the subunits was observed. However, to completely understand the role of the conserved motif, mutations within the CP and subsequent electrophysiological analyses are required. Especially, the effect of mutations of the “leucine-zipper” residues L122 and L129 should be investigated. Also the possible salt-bridge between aspartic acid and arginine of neighboring subunits provide a potential target for engineering. If one of the mutation-scenarios leads to a phenotype comparable to the truncation mutant Δ110-136 MscL, with the characteristic prolonged open state dwell-times, it would provide additional support for the stable bundle hypothesis and shed light on the role of the individual residues maintaining it by (i) hydrophobic effects, (ii) salt-bridges, or (iii) a combination of both.