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The conserved carboxy-terminus of the MscS mechanosensitive channel is not essential but increases stability and activity

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Abstract **The *Escherichia coli* MscS mechanosensitive channel protein has a distinct domain structure that terminates in a conserved seven-strand β barrel. This distinctive feature suggested it could be a critical determinant of channel stability and activity. Measurements on a protein deleted for the base of the vestibule and the β barrel (residues 266–286) suggested that the modified channel had reduced activity. However, induction of the mutant protein resulted in membrane protein accumulation equivalent to wild type and a physiologically functional channel. In patch clamp analysis the activity profile was similar to wild type but reduced numbers of channel were seen per patch, suggesting reduced assembly or stability of the mutant protein. The mutant channel exhibited a subtle change in character – channels did not re-open after full desensitization. Thus the immediate carboxy-terminus (residues 266–286) is not essential for MscS gating but improves stability and activity and is required for recovery of channel activity after desensitization.**

Keywords: MscS; Mechanosensitive channel; β Barrel; Electrophysiology; Desensitization; *Escherichia coli*

1. Introduction

Mechanosensitive (MS) channels perform a remarkable transition between the closed and open states in response to membrane deformation that arises from a sudden increase in the transmembrane (TM) pressure [1–4]. Proteins that form these channels must have the capacity to maintain a tight seal such that in the closed state there is no ion conduction. However in the open state the pores have been calculated at between 14 and 35 Å diameter, respectively, for *Escherichia coli* MscS and MscL, allowing conductance values of 1 nS or greater [5,6]. Considerable interest has thus been expressed in the channel structures and the structural transitions that permit rapid opening of such large pores [7,8]. The crystal structure for MscL from *Mycobacterium tuberculosis* has been solved and extensive analysis of the gating performed using biochemical and genetic approaches. The MscL channel has been shown to exhibit substates but does not show significant desensitization during the application of sustained pressure. However, the gating transition is quite well understood [9–13].

In contrast to MscL, the gating transition in MscS is poorly understood. The structure of the MscS channel was solved at 3.9 Å [8]. It is a homoheptamer, each subunit consisting of two major domains: the amino-terminal membrane domain and the carboxy-terminal cytoplasmic domain. In *E. coli* each subunit has three TM helices. TM3 lines the channel pore [8,14] and contains predominantly hydrophobic residues with L_{109} and L_{105} making an effective seal [14]. Amino acid substitutions that alter the packing of TM3 helices either facilitate or inhibit channel gating depending on the exact position of the residue mutated (Edwards et al., unpublished data). This has led to the proposal that MscS gating involves rotation of the TM3 helices combined with a slight outward tilt at the periplasmic end. This rotation is transmitted to the cytoplasmic domain via an extension of TM3 which bends around G_{113} (*E. coli* numbering) such that the amphipathic region of the helix (G_{113} to F_{127}) lies parallel to the membrane surface (Fig. 1A) [15]. The large (~17 kDa) cytoplasmic carboxy-terminal domain exhibits three major sub-domains [8]. A β sub-domain (residues 132–177) is linked to a mixed αβ sub-domain (residues 188–265) and the protein terminates in a β barrel (residues 271–280; residues 281–286 were not resolved in the crystal structure). The cytoplasmic domain creates a large vestibule that is perforated by seven lateral portals created by the junctions of the β and αβ sub-domains and an axial portal formed by the β barrel. This complex structure may be partially explained by the possibility that the lateral 14 Å portals serve as selectivity filters, which could in turn explain the anionic preference of MscS [8,16]. The length of the carboxy-terminal domain and the sequence that forms the base of the vestibule and the barrel are strongly conserved (Fig. 1B).

Evidence for a significant structural re-organization of the cytoplasmic domain during gating comes from two separate studies. Firstly, in patch clamp assays, opening the $MscSH_6$ channel in response to pressure could be prevented by Ni^{2+}. This did not occur if the His-tag was excluded, thus implying that Ni-coordination of the tags inhibited a structural transition [17]. Secondly, our biochemical analysis showed that single cysteine substitutions that were separated by up to 33 Å in the crystal structure could form disulphide bridges when the channel was in the closed state [18]. Further, cross-linking of single cysteine residues with *o*-phenylenedimaleimide readily

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Abbreviations: MS, mechanosensitive; TM, transmembrane

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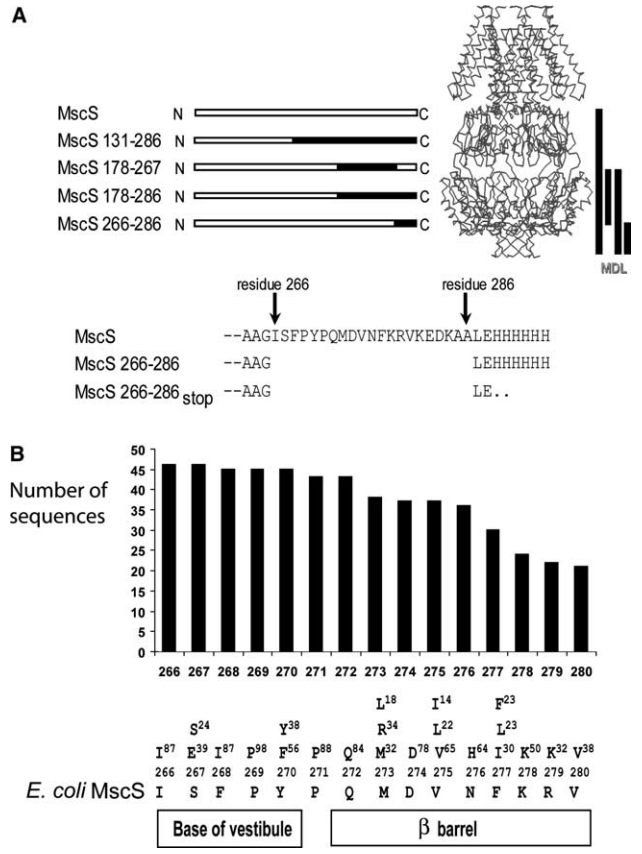

Fig. 1. Deletion mutants eliminated specific sub-domains of the carboxy-terminus. (A) Crystal structure of the MscS protein (drawn in Chime [25]) with horizontal bars on the left-hand side diagrammatically representing the deleted sequences (black bars). N and C denote amino-terminal and carboxy-terminal ends, respectively, of the carboxy-terminal domain. Vertical bars to right of crystal structure indicate the deleted regions only. Amino acid sequences showing the last 24 residues of wild type MscS and their connection to a carboxy-terminal His-tag and the corresponding sequences of the $\Delta 266–286H_6$ and $\Delta 266–286_{stop}$ mutant proteins ('.' indicates a stop codon). (B) The sequence corresponding to the carboxy-terminal domain of *E. coli* MscS (residues 126–286) were used in a BLAST [26] search of all microbial genomes. After elimination of duplicate sequences, 46 homologues were aligned and the graph shows the number of sequences that contain a residue at each position corresponding to the base of the vestibule and the β barrel (residues 266–280) (upper panel) and the conservation at each position (lower panel). The values as superscripts are the percentage of homologues with that residue.

generated multimeric forms higher than the dimer, consistent with oligomer stabilization. The most dramatic effects were seen with S267C, which lies at the junction of the αβ sub-domain and the terminal β barrel. In the crystal structure, which depicts the protein in an open configuration (either partially or completely), S267C residues should be sterically inhibited from reacting with each other. However, in the closed structure they readily react and form stable structures up to the heptamer. The complex nature of the carboxy-terminal domain led us to re-examine its importance for channel stability and activity. Deletions with termini at the junctions between the different cytoplasmic sub-domains were created. Here we show that while three specific sets of deletions impaired stability of the protein in the membrane, removal of residues 266–286, which includes the terminal β barrel (residues 271–280), produced a stably expressed, active channel. However, the mutant protein had subtle changes in assembly or stability, manifested by altered numbers of channels in membrane patches and failure of the channel to re-open after desensitization. The data are consistent with the carboxy-terminus being non-essential but enabling organization of the protein and facilitating cycles of activity.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli strains MJF465 ($MscL^-MscS^-MscK^-$), PB114 ($MscS^-MscK^-YjeP^-RecA^-$) and JM109 were used throughout this study and have been described previously [19,20]. Plasmids pTrc99A and $pMscSH_6$ have been described previously [14,19].

2.2. Mutant creation

Plasmids pMscSΔ131–286, pMscSΔ178–267, pMscSΔ178–286 and pMscSΔ266–286 were created by inserting *Xho*I sites at the relevant position by PCR, digestion of the resultant plasmids with *Xho*I to remove the sequence between the mutation and the *Xho*I site immediately 5′ to the His-tag sequence and re-ligating the plasmid. For the deletion that has its termini internal to the MscS protein, the *Xho*I site adjacent to the His-tag was first removed by mutagenesis, retaining the native amino acid sequence, and new *Xho*I sites created at the appropriate positions for the required deletion. To create $pMscS\Delta 266–286_{stop}$ the first two residues of the His-tag were converted into stop codons, simultaneously removing the *Xho*I site, using pMscSΔ266–286 as template. All mutant plasmids were sequenced on both strands at least twice, using the BIG-DYE reaction mix (Amersham) as instructed by the supplier.

2.3. Downshock protocol

Survival of osmotic downshock was essentially as described previously [19] for cells grown in minimal medium with the following modifications: all survival experiments were performed using transformants of MJF465, the culture was adapted to high osmolarity by growth in the presence of 0.3 M NaCl and downshock was a 1:20 dilution into distilled water (shock) or medium containing 0.3 M NaCl (control). Data are reported as means ± S.D.

2.4. Membrane preparations and Western blots

Western blots were performed on membrane preparations from IPTG-induced cells (0.3 mM, 30 min) as described previously [14]. Membrane proteins (15 μg per track) were separated using pre-formed 4–12% SDS–PAGE gels (Novex) and transferred to nitrocellulose. The primary antisera was rabbit anti-YggB3 [14]. The secondary antibody was peroxidase-conjugated anti-mouse IgG (whole molecule). Images were developed by ECL (Pierce) on photographic film (Kodak).

2.5. Electrophysiology

Patch clamp recordings were conducted on giant protoplasts as described previously [21] using strain PB114 [20]. Excised, inside-out patches were analysed at −20 mV with pipette and bath solutions containing 200 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$ and 5 mM HEPES buffer at pH 7. All data were acquired at a sampling rate of 10 kHz with 5 kHz filtration using an AxoPatch 200 amplifier and pClamp software (Axon). The pressure threshold for activation of the MscS channels, with respect to the activation threshold of MscL, was determined as described previously [20,22,23]. All measurements have been conducted on patches derived from at least two protoplast preparations. Pressure ratios are given as means ± S.E.M. Student's *t* test was used for determining significance.

2.6. Materials

Media components were purchased from Oxoid and all salts from BDH. Restriction enzymes were obtained from Roche or Promega, and pre-cast Novex SDS–polyacrylamide gels were from Invitrogen-Life Technologies. Primary and secondary antibodies came from Sigma, the Supersignal Dura substrate from Perbio.

3. Results and discussion

3.1. Expression and activity of MscS deletion mutants

Mutants were created such that their deleted sequence corresponded with the junctions between the different structural elements of the carboxy-terminal domain (Fig. 1A). Western blots of membranes indicated that only the full-length (wild type) protein and the $\Delta 266–286H_6$ protein, which was the smallest of the deletions, were stably incorporated into the membrane (Fig. 2A). Proteins with larger deletions were not observed in membranes from induced cells (Fig. 2A). Previously, a carboxy-terminal His-tag was shown to affect the activity of the MscS channel in the presence of Ni^{2+} ions [17]. Thus a new construct was generated, $\Delta 266–286_{stop}$, that eliminated the His-tag sequence (Fig. 1A). These two mutant proteins (with and without His-tag) expressed to wild type levels with induction (Fig. 2A). Channel activity can be detected physiologically by the suppression of the hypoosmotic shock-induced lysis phenotype observed in strain MJF465 [19]. The plasmid construct allows basal expression of the cloned MscS channels and higher levels of expression were achieved by 30 min incubation with IPTG (0.3 mM). This assay system allows subtle changes in channel stability or activity to be detected [14]. No protection was afforded by expression of the larger deletion mutants (percentage survival after induction with IPTG: 2.5 ± 2, 2 ± 1.5, 7 ± 10 for MJF465 expressing MscSΔ131–$286H_6$, MscSΔ178–$286H_6$ or MscSΔ178–$267H_6$, respectively, [$n = 4$], similar to $4 \pm 2\%$ for MJF465 alone) but the $\Delta 266–286H_6$ and $\Delta 266–286_{stop}$ mutants possessed activity (Fig. 2B). At basal levels, the $\Delta 266–286H_6$ channels protected cells similar to full-length MscS, whereas $\Delta 266–286_{stop}$ channels appeared to display lower activity (Fig. 2B). When the proteins were over-expressed, which resulted in equivalent levels of membrane accumulation (Fig. 2A), protection increased to ~100% for all three proteins (Fig. 2B). These data are consistent with deletion of the extreme carboxy-terminus, including the β barrel, lowering either assembly or stability such that without induction there are fewer channels present in the membrane.

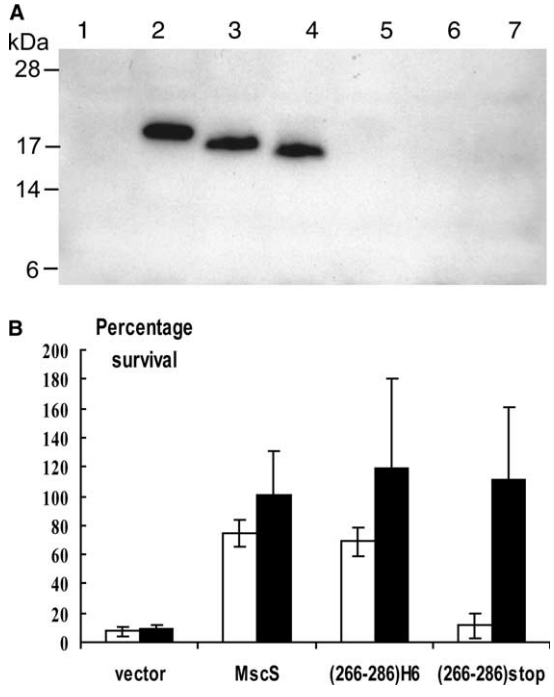

Fig. 2. Over-expressed $\Delta 266–286H_6$ and $\Delta 266–286_{stop}$ mutant proteins exhibit stability and activity. (A) Membrane expression of channel proteins was identified by immuno-staining with the anti-MscS antibody [14]; (Key: 1, vector only; 2, MscS; 3, $\Delta 266–286H_6$; 4, $\Delta 266–286_{stop}$; 5, $\Delta 131–286H_6$; 6, $\Delta 178–267H_6$; 7, $\Delta 178–286H_6$). (B) The ability of the MscS deletion plasmids to survive hypoosmotic shock was assayed as described in Section 2. Percentage survival is shown for uninduced (white bars) and IPTG-induced (0.3 mM IPTG for 30 min prior to assay; black bars) cultures. Data shown are the means ± S.D. of a minimum of four experiments.

3.2. Electrophysiology of carboxy-terminal deletion mutants

The survival data suggested that the deletion mutants might exhibit altered electrophysiology. Protoplasts of PB114 expressing uninduced wild type, $\Delta 266–286H_6$ or $\Delta 266–286_{stop}$ channel proteins were analysed (Fig. 3). Initial recordings showed the two mutant proteins formed channels that were effectively indistinguishable from the wild type. The channels displayed the characteristic pattern of opening and closure during pressure application that is typical of MscS activity [24]. Transient pressure applications (10–20 s duration) resulted in multiple channels opening and these then closed on release of pressure, irrespective of which channel was being examined. Many cycles of such transient pressure applications could be undertaken for all three channel types and resulted in channel openings with no loss of activity. Further analysis indicated slightly more pressure was required to open the $\Delta 266–286_{stop}$ channels ($P_{MscL}:P_{MscS} = 1.59 \pm 0.03$ ($n = 14$), 1.53 ± 0.02 ($n = 12$) and 1.46 ± 0.02 ($n = 22$) for wild type, $\Delta 266–286H_6$ and $\Delta 266–286_{stop}$, respectively, $P < 0.05$ for wild type vs. $\Delta 266–286_{stop}$). MscS channels exhibit desensitization when maintained under pressure, a feature not shared by other members of this family that have been assayed [19,21]. We

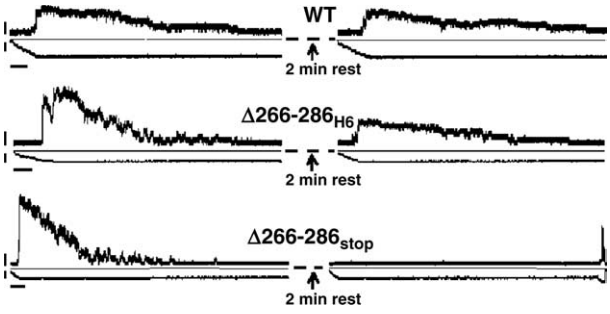

Fig. 3. Electrophysiological comparison of wild type and mutant MscS channels. Protoplast membrane patches expressing wild type (WT), $\Delta 266–286H_6$ or $\Delta 266–286_{stop}$ plasmids were subjected to sufficient pressure to open the MscS channels present, at which point the pressure was clamped to allow the channels to desensitize and close (left-hand traces). After a 2 min rest pressure was applied as before and channel activity recorded (right-hand traces). It should be noted that for the $\Delta 266–286_{stop}$ mutant, MscS channels did not re-open at their previous gating-pressure after full desensitization, whether low or high numbers were present initially; pressure was clamped at this level before increasing its magnitude until MscL channels were observed (as illustrated at the end of the right-hand recording for this mutant). Downward deflections (scale bars ≡ 100 mmHg) represent the pressure levels and upward profiles (scale bars ≡ 100 pA) are the channel openings in each trace. Horizontal time scale bars represent 5000 ms for both wild type traces, 4000 ms for both $\Delta 266–286H_6$ traces and 10 000 ms for both $\Delta 266–286_{stop}$ traces.

Table 1
Analysis of MscS channel activity recovery after desensitization

Channel	Channels/patch initial activity		Channels/patch recovered activity		Percentage recovery[a]
	Mean and S.D.	Range	Mean and S.D.	Range	
Wild type	13 ± 10 ($n = 11$)	3–30	10 ± 8 ($n = 11$)	3–27	82 ± 20
$\Delta 266–286H_6$	11 ± 5 ($n = 9$)	3–17	5 ± 2 ($n = 9$)	3–8	55 ± 20[c]
$\Delta 266–286_{stop}$	5 ± 5[b] ($n = 15$)	1–23	0 ($n = 15$)	0	0

[a] The percentage recovery of channel activity following desensitization and a 2 min rest was calculated for each patch based upon the numbers of channels observed before and after the desensitization test (see text).
[b] The number of channels in $\Delta 266–286_{stop}$ patches was significantly lower compared to wild type and $\Delta 266–286H_6$ containing patches ($P < 0.05$).
[c] The difference between wild type and $\Delta 266–286H_6$ channel recovery after a 2 min rest was significant ($P < 0.05$).

found that loss of the carboxy-terminus did not significantly affect the desensitization property of the channels during the first sustained pressure test (Fig. 3). Channel activity decayed in a similar manner to wild type. After a 2 min rest, pressure was re-applied to the patches and the full-length ($n = 11$) and $\Delta 266–286H_6$ ($n = 9$) channels were observed to reversibly desensitize (Fig. 3). The initial average activity of MscS corresponded to approx. 13 channels per patch for wild type and 11 channels for the $\Delta 266–286H_6$ protein; the range of values was similar ($P > 0.05$) for patches from both strains (Table 1). After desensitization the number of channels recovered was seen to depend on the length of the rest period. After a 2 min rest, >80% of wild type channels were recovered, whereas 55% of channels were re-activated for the $\Delta 266–286H_6$ after 2 min (Table 1) and >95% after a 10 min rest ($n = 3$; data not shown). In contrast, for $\Delta 266–286_{stop}$ the average number of channels per patch was 3–5 (range 1–23 channels; $n = 15$) and channels were not observed following a 2 min rest after full desensitization in any of these patches (Table 1; Fig. 3). Even after a 10 min rest, no channels were recovered at pressures that previously opened MscS ($n = 5$; data not shown). However, for the majority of such patches single channels with the same conductance as MscS opened simultaneously with MscL (i.e., these channels had $P_{MscL}:P_{MscS} \sim 1$). No such change in pressure was required to re-open either wild type or $\Delta 266–286H_6$ channels after desensitization (data not shown). Thus, although lower numbers of mutant channels were inserted into the membrane by cells expressing the $\Delta 266–286_{stop}$ construct, which explains the reduced protection afforded in the survival assay without induction (Fig. 2B), the channels clearly gated in response to pressure and desensitized in a manner similar to wild type. However, re-organization of this mutant channel after desensitization was prevented.

The desensitization phenomenon is poorly understood. We routinely observe that, after MscS closure under sustained pressure (desensitization), some or all channels will always re-open upon the re-application of pressure (i.e., it is a reversible process). It is not clear if this is a physiologically important phenomenon, but the greater complexity of the gating of MscS compared to MscL may require additional inactivation mechanisms. For example, the open probability of MscS is increased by depolarization [24] and this may pose problems for cells that have been depolarized either by the action of MscM opening, which occurs at lower pressures than MscS, or the actions of other ion channels. The inability of the $\Delta 266–286_{stop}$ channel to exhibit the restoration of proper channel activity after desensitization shows that the conformational change required for reactivation is not attainable and confirms that desensitization leads to a unique conformation in MscS that is distinct from both the open and the closed states. Interestingly, recovery of channel activity after desensitization was possible in the presence of a carboxy-teminal His-tag, which may reflect a partial ability of this sequence (LEHHHHHH) to mimic the base of the vestibule allowing the mutant protein to achieve a conformation after desensitization from which it can again open on imposition of pressure (Fig. 3; Table 1).

The MscS crystal structure depicts an open state in which the carboxy-terminal domains are brought together in a β barrel. This region is conserved, both in length and sequence, in close homologues of the *E. coli* MscS (Fig. 1B), which suggested that it might be essential. In this study we have shown that the absence of the base of the vestibule and the β barrel in a mutant channel lowers the number of active channels in the membrane and this is reflected in the lower protection against hypoosmotic shock observed with cells not induced for channel expression. The mutant channel also exhibits a slight resistance to pressure-induced opening and failure to recover after desensitization. We have previously proposed that during the gating process major structural rearrangements occur in the carboxy-terminal domain [18]. Here we have demonstrated that the base of the vestibule and the conserved terminal β barrel are not essential for MscS activity but that their presence increases either assembly or stability and facilitates conformational change(s) in the recovery from the desensitized state.

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