Three Routes To Modulate the Pore Size of the MscL Channel/Nanovalve

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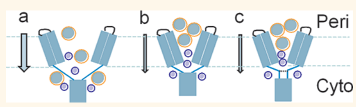
ABSTRACT MscL is a bacterial mechanosensitive channel that protects cells from lysis upon acute decrease in external osmotic environment. It is one of the best characterized mechanosensors known, thus serving as a paradigm of how such molecules sense and respond to stimuli. In addition, the fact that it can be genetically modified, expressed, isolated, and manipulated has led to its proposed use as a triggered nanovalve for various functions including sensors within microelectronic array chips, as well as vesicular-based targeted drug release. X-ray crystallography reveals a homopentameric complex with each subunit containing two transmembrane α-helices (TM1 and TM2) and a single carboxyl terminal α-helix arranging within the complex to form a 5-fold cytoplasmic bundle (CB), whose function and stability remain unclear. In this study, we show three routes that throttle the open channel conductance. When the linker between the TM2 and CB domain is shortened by deletions or constrained by either cross-linking or heavy metal coordination, the conductance of the channel is reduced; in the later two cases, even reversibly. While they have implications for the stability of the CB, these data also provide routes for engineering MscL sensors that are more versatile for potential nanotech devices.



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The mechanosensitive channel of large conductance, MscL, is a bacterial channel located in the cytoplasmic membrane that protects cells from lysis upon acute decrease in external osmotic environment by releasing cytoplasmic osmolytes.[1] This channel was first identified in *E. coli*, and most functional studies have been undertaken in this species. A MscL homologue from *Mycobacterium tuberculosis*, which has 67% similarity, has been resolved by X-ray crystallography and shown to be a homopentamer, with each subunit containing two transmembrane α helices (TM1 and TM2), of which TM1 forms the pore of the channel and TM2 is exposed to the lipid bilayer.[2] Although a more recent crystal structure of MscL from *S. aureus* shows a tetrameric complex,[3] this appears to be a detergent-specific oligomeric organization not reflecting a physiological state; *in vivo* essentially all channels are pentameric.[4,5] The pentameric channel opens by the expansion of both TM1 and TM2 in response to tension in cell membrane.[6−11] The channel also contains a single carboxyl terminal α helix from each subunit that together assemble into a 5-helix cytoplasmic bundle (CB).[2] It remains unclear what the function of the CB is, and whether or to what extent the bundle is dissociated during channel gating.

Previous studies have shown that the *E. coli* MscL channel has a very large pore size, greater than 30 Å,[12] which has caused some researchers to speculate that it could be used in nanodevices. Indeed, it has many properties that would make it ideal for use as a triggered nanovalve in such devices. It can be translated *in vitro*[13] or synthetically synthesized, reconstituted into lipids, and yet spontaneously assemble into a functional complex.[14] Since the introduction of charges into the channel pore lumen can gate the channel in the absence of membrane tension,[7,15−18] *E. coli* MscL has been engineered into controllable nanovalves that detect alternative modalities including light[19] and pH.[20] The channel has been shown to be functional in vesicular-release devices,[19−21] as well as in an engineered microelectronic array chip;[22] its large pore size yields a robust response in such devices. However, for some purposes a smaller or adjustable pore size would be advantageous.

The data presented within this study on *E. coli* MscL have two aspects: first, they resolve the issue of whether the CB disassociates upon normal channel opening, and second, they demonstrate that the MscL nanopore response can be effectively and reversibly modulated, which would have advantages in an assortment of potential nanodevices.

RESULTS AND DISCUSSION

Disassociation of the c-Terminal α Helical Bundle Is Not Required for Normal Gating and Conductance of the MscL Channel. As shown in Figure 1a, the

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X-ray crystal structure of the *M. tuberculosis* MscL channel shows a pentameric structure in which the five subunits, at the c-terminal, form an α helical cytoplasmic terminal bundle (CB). The conformation, functional role, and stability of the CB of the MscL channel have remained controversial. We reasoned that if the CB was indeed a helical bundle and was stable even upon gating, the sieve-like structure suggested avenues for controlling channel pore size and conductance by constraints and deletions within this area.

The CB was originally revealed in the crystal structure of the *M. tuberculosis* MscL,[2] but it adopted what appeared to be a nonstable confirmation with negatively charged residues facing each other. Subsequently, Anishkin *et al*. proposed a more stable conformation for the CB based on the crystal structure of the pentameric cartilage oligomeric matrix protein (COMP), which has a consensus motif, ALQDVRELLR, similar to that of the MscL CB, LLxEIRDLLK.[23] The COMP crystal structure showed a strongly amphipathic helix in which all apolar residues are packed inside the 5-fold bundle. In an attempt to address the correctness and the stability of this proposed structure for the CB, Anishkin *et al*. mutated the pairs of leucines predicted to face each other to cysteines within the consensus motif; these formed cross bridges between subunits, as anticipated from the authors' proposed structure, yet normal channel activity was observed.[23] The authors interpreted these data as suggesting not only that their proposed structure was correct, but that the CB remained intact upon gating. Subsequently, a re-evaluation of the MscL crystal structure[24] confirmed the more stable conformation of the CB. However, a site-directed spin labeling study on *E. coli* MscL revealed a significant difference in the structure of the end of TM2, which was explained by several possibilities, including that the c-terminus of each channel subunit might not assemble into CB but, instead, fold toward the membrane interface establishing specific tertiary contacts with TM2.[25] In addition, a more recent study using atomic force microscopy noted that the bundle appeared to be missing in a spontaneously opened MscL mutant, suggesting that the CB was not as stable as predicted and did indeed disassociate upon gating.[26] In critically evaluating the Anishkin *et al*. study, we noted that the patch clamp traces were obtained at threshold, rather than saturating, stimulus conditions, but patch clamp is exquisitely sensitive, detecting single molecular events, while the cysteine cross-linking at these sites is extremely inefficient. It therefore seemed possible that the activities observed in the Anishkin study could simply be the channels that contained little or no cross-linking.

We therefore performed experiments where saturating stimulus was applied to a mutant to determine whether the opening of this CB is required for normal

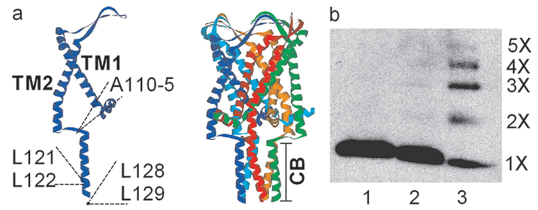

Figure 1. ***In vivo*** **cross-linking of cytoplasmic terminal bundle (CB) of MscL L121−122C/L128−129C mutant. (a) The side view of X-ray crystal structure of MscL from *Mycobacterium tuberculosis* (PDB code 2OAR). A single subunit of MscL channel is shown on the left and is also highlighted as dark gray in a homopentameric channel (right). Each subunit contains two transmembrane α-helices (TM1 and TM2) and one cytoplasmic a-helix, which assemble into a 5-fold cytoplasmic terminal bundle (CB). In the middle of the cytoplasmic linker, the equivalent amino acids of *E. coli* (A110−115) that were either mutated or deleted for this study are shown, as well as the location of the mutated leucines within the bundle. Note that the *E. coli* 128 and 129 L are not observed in the *M. tuberculosis* structure. (b) Western blot showing that disulfide bridges of a MscL tetra cysteine mutant (L121−122C/L128−129C) lead to cross-linking of channel subunits. Before loading with nonreducing Laemmli sample buffer, cells expressing MscL L121−122C/L128−129C mutant were grown in high osmolarity (lane 1), some of which were then osmoticaly downshocked (lane 2), or osmoticaly downshocked in the presence of Cu-phenanthroline (lane 3), with the latter having a ladder of monomer through pentamer (1X−5X) formed by disulfide bridging between different subunits of the complex.**

E. coli MscL channel gating and conductance; we mutated all four leucine residues in the amphipathic motif, which are predicted to face each other,[23,24] to cysteines (L121−122C and L128−129C). This allowed for stabilization of the CB by the formation of disulfide bridges. As seen in Figure 1b, and consistent with a previous study,[23] the extent of cross-linking in individual MscL complexes varies, giving a ladder of MscL monomers to pentamers in a nonreduced SDS PAGE gel as visualized by Western. Given that patch clamp records single molecular events, it is possible to observe a minority of channels, presumably only those that contain no disulfide bridges and thus have normal sensitivity and gating properties. To control for this possibility, we measured the total current from all channels within the patch by using a saturating stimulus. In this experiment, the total MscL current under ambient conditions was 5,176 pA. After addition of oxidizing agent Cu-phenanthroline of 1 mM in bath solution for 20 min, the total MscL channel current in the same path was 5,240 pA, demonstrating that few or none of the channels were locked closed or in a substate. Upon application of 10 mM DTT for 20 min to bath solution, the total MscL channel current was measured to be 5080 pA, demonstrating that no channels were revealed under reduced conditions. Cu-phenanthroline and DTT treatment also did not appear to change the mechanosensitivity of MscL L121−122C, L128−129C; the ratio of pMscL/pMscS threshold, as described previously[27] and in the Methods section, was 1.5 ± 0.1 before and after Cu-phenanthroline treatment ($p > 0.05$, paired

TABLE 1. Summary of Single Channel Current and Mechanosensitivity of MscL Channels Tested in This Paper with the Different Experiments Organized into Four Groups

		single channel current (pA)	*n*	mechanosensitivity[c]	*n*
		Pressure Gated Experiments			
WT		84.7 ± 1.7	12	1.5 ± 0.0	12
Δ110–112		73.1 ± 1.2**	5	1.5 ± 0.1	5
Δ110–115		<30	19	1.8 ± 0.1***	14
A110–112C		49.1 ± 5.0***	15	1.7 ± 0.1	31
		MTSET$^+$ Gated Experiments			
G22C		19.5 ± 1.9	4	0	4
G22C/ Δ110–115		9.5 ± 1.8**	7	0	7
		Pressure Gated REDOX Experiments			
A110–112C	Control	49.1 ± 5.0	15	1.7 ± 0.1	4
	DTT	70.4 ± 2.6***	15	1.9 ± 0.2	4
		Pressure Gated $ZnCl_2$ Treatment Experiments			
A110H	Control	74.9 ± 1.7	6	138.3 ± 13.8	6
	$ZnCl_2$	35.7 ± 2.1***	6	133.5 ± 13.1	6
A112H	Control	80.2 ± 3.6	5	136.3 ± 14.0	5
	$ZnCl_2$	43.1 ± 3.9***	5	150.2 ± 16.9*	5

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$ by *t* text when compared with wild type (WT) MscL, G22C (group 2), or the corresponding controls (groups 3 and 4). [c] Mechanosensitivity is expressed in pMscL/pMscS for most mutant and wild type channels except in pMscL for A110H and A112H mutants, as described in the Methods section.

t test, *n* = 7). These results definitively show that disassociation of the CB is not required for normal gating and conductance of the MscL channel.

Deletions within the TM2/CB Linker Lead to Channels with Decreased Conductance. Although it is clear that disassociation of the CB is not *required* for normal gating, it is still useful to know whether the CB, when not constrained, normally dissociates during channel gating. If the CB does not disassociate, then deletions within the TM2/CB linker of *E. coli* MscL, which is similar to length as that shown for the *M. tuberculosis* structure in Figure 1, should lead to constraints of the TM2 movement or decreases in the size of the "sieve", thus modifying the membrane tension mechanosensitivity threshold or lowering conductance. To test this hypothesis, we kept the CB intact and deleted different ranges of amino acids from the TM2/CB linker of *E. coli* MscL. All point histogram analyses revealed that single channel currents from MscL Δ110–112 were 73.1 ± 1.2 pA, a range significantly lower than that of wild type (WT) MscL channel (84.7 ± 1.7 pA) (Table 1). The MscL Δ110–115 channel, which had a more significant deletion, had a further attenuated single channel current with most of the single channel openings below 30 pA (Table 1). The pMscL/pMscS of MscL Δ110–112 and MscL Δ110–115 were 1.5 ± 0.1 and 1.8 ± 0.1, respectively, with the latter significantly increased relative to that for wild type MscL (1.5 ± 0.0) (Table 1).

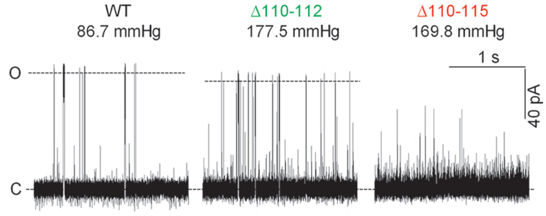

Figure 2. Deletions in TM2/CB linker of MscL decrease its current. "O" and "C" refer to full opening and closing of the channel, respectively. Pressure applied to patch pipet is shown in mmHg. As shown, compared with single channel current of wild type MscL (WT), MscL with 110–112 deletion (Δ110–112) has slightly decreased current, and the current of the MscL with the 110–115 deletion (Δ110–115) is further reduced and has a short open time, or `flickery' behavior.

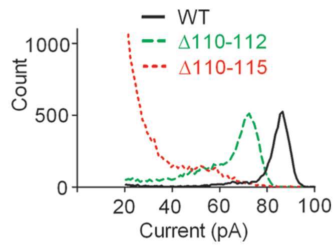

Figure 3. All point histogram of the TM2/CB linker deletions. As shown, the single channel current of wild type MscL (WT) is 87pA, while that of MscL Δ110–112 is decreased to 73pA. Because of the short open times observed for MscL Δ110–115, the peak opening currents could not easily be measured, but most of the channel openings are below 30 pA.

Hence, the Δ110–115 mutant was significantly less sensitive to membrane tension. Representative current traces and all point histograms of wild type MscL, MscL Δ110–112 and Δ110–115 are shown in Figures 2 and 3. These data contrast studies in which half of the TM2/CB linker as well as the entire CB were deleted. For example, the MscL Δ110–136 deletion mutant has no apparent change in either mechanosensitivity or conductance.[23,27,28] More recently, it has been shown that a similar Δ95–119 *S. aureus* truncated channel assembles into a normal pentameric complex *in vivo*[4,5] and effects relatively normal channel activity.[3,5] Together, these data demonstrate that although the CB is not required for normal assembly or gating, if it is present, the CB is quite stable and does not dissociate during the normal gating process; these data also reveal a novel way to systematically control MscL channel pore size and conductance.

Engineering a Triggered Nanovalve with a Smaller Pore Size. The MscL G22C mutant has been reported to be able to be used as a triggered nanovalve in liposomes that could modulate the release of a liposome payload.[19,20] Briefly, a charge in the pore can gate the channel; thus, the G22C MscL mutant can be gated by the positively charged thiol reactive agent MTSET$^+$. We therefore combined the mutations of G22C and Δ110–115 in

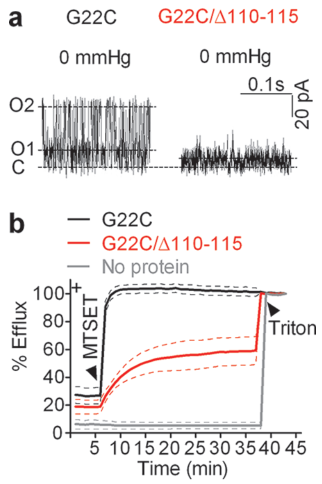

Figure 4. Engineering and testing of a MscL nanovalve with a decreased pore size. (a) Patch clamp recordings show the spontaneous single channel opening of G22C (left) and G22C/Δ110−115 (right) after MTSET$^+$ treatment. "O1" and "C" refer to main opening and closing of the channel, respectively, and "O2" refers to a higher level opening of the channel; note that no pressure was needed to gate either channel. (b) Percentage calcein release from vesicles reconstituted with G22C (black line), G22C/Δ110−115 (red line), or without MscL protein (gray line). The dashed lines show the standard derivation of fluorescence signal from three independent experiments. The arrows indicate the addition of MTSET$^+$, followed by Triton X-100 detergent to measure the 100% calcein content.

order to engineer a triggered MscL nanovalve with throttled currents. As shown in Figure 4a, the resulting G22C/Δ110−112 mutant had spontaneous openings after MTSET$^+$ treatment that were similar to the MscL G22C alone. All point histogram analyses showed that, when gated by MTSET$^+$, single channels of G22C with the Δ110−115 deletion indeed opened primarily to allow lower currents of 9.5 ± 1.8 pA, while G22C opened mostly to allow larger currents of 19.5 ± 1.9 pA (Table 1). Moreover openings of G22C MscL at 56.5 ± 1.5 pA could also be seen, but were not prominent in the deletion mutant. These two mutants were observed to open at 74.0 ± 1.0 pA and 76.0 ± 1.0 pA only in very rare instances. The representative traces of the spontaneous opening of MscL G22C and G22C/Δ110−115 are shown in Figure 4a. These data are consistent with a stable CB: the shorter the cytoplasmic linker, the higher the restriction imposed on the TM2 movement or the smaller in sieve size, and thus the smaller and less stable the open pore.

The small conductance of G22C with Δ110−115 suggests a small channel pore size and likely a decreased ability to translocate larger compounds. We therefore performed a calcein efflux assay on liposomes reconstituted with the G22C/Δ110−115 mutant MscL proteins, as previously described.[19−21] Liposomes loaded with 50 mM calcein have a low fluorescence signal due to self-quenching of the dye at this concentration. When calcein is released from liposomes into the bulk solution, however, its apparent fluorescence increases.

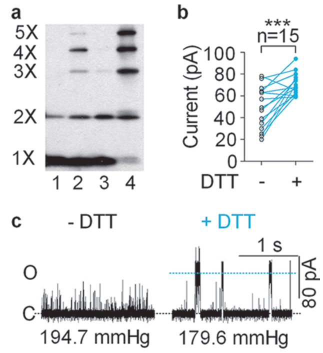

Figure 5. Cysteine cross-linking decreases MscL A110−112 current. (a) Western blot showing that disulfide bridges of a MscL triple cysteine mutant (A110−112C) lead to cross-linking of channel subunits. Before being loaded with nonreducing Laemmli sample buffer, cells expressing MscL A110−112C mutant were grown in high osmolarity (lane 1), some of which were then osmoticaly downshocked (lane 2), or treated with oxidizing agent Cu-phenanthroline (lane 3), or osmoticaly downshocked in the presence of Cu-phenanthroline (lane 4). A ladder of monomer through pentamer (1X−5X) is formed by cross-linking between different subunits. As shown, shock plus Cu-phenanthroline treatment leads to the most pronounced formation of cross-linking among channel subunits. (b) Change in the current of MscL A110−112C mutant before and after DTT treatment. Data were pooled from 15 patch clamp recordings. (***) $p < 0.001$ by two-tailed and paired *t* test. (c) One representative patch clamp recording of single channel opening of MscL A110−112C before (left) and after (right) DTT treatment, which serves as a reducing agent to break the disulfide bridges formed between two cysteines. "O" and "C" refer to full opening and closing of the channel, respectively. Pressure applied to patch pipet is shown in mmHg.

As shown in Figure 4b, G22C/Δ110−115 MscL had a much reduced calcein efflux compared with G22C MscL consistent with pore size reduction; no calcein efflux was observed from liposomes not reconstituted with the MscL protein.

Disulfide Cross-Linking of the TM2/CB Linker Decreases Channel Conductance. The TM2/CB linker is potentially a highly dynamic region; thus constraining this region should lead to a channel with decreased conductance. We therefore mutated the three alanines at positions 110−112, which are approximately in the middle of the TM2/CB linker, to cysteines. The formation of disulfide cross-links between MscL A110−112C channel subunits was confirmed by Western blot (Figure 5), where dimers, trimers, tetramers and pentamers were measured. For these experiments *E. coli* had a reducing environment in the cytoplasm, which was maintained by glutathione and thioredoxin systems.[29] Thus, as previously described,[30] when bacteria released these endogenous reducing agents upon osmotic shock, more cross-linking between different subunits was observed. As expected, osmotic downshock itself increased cross-linking and, together with Cu-phenanthroline treatment, it showed the most pronounced cross-linking (Figure 5a).

Patch clamp recordings under ambient conditions showed that MscL A110−112C opens under normal levels of stimuli, but does not open to a full and normal unitary conductance. However, after DTT was added to the bath solution to cleave the disulfide bridges, the conductance of the channel significantly increased (Figure 5b). The variance observed for the conductance of MscL A110−112C is presumably due to the different amounts of cross-linking within individual channels. Representative traces of MscL A110−112C before and after DTT treatment is shown in Figure 5c. DTT treatment did not alter the mechanosensitivity of MscL A110−112C channel (pMscL/pMscS threshold was 1.7 ± 0.1 before DTT treatment and 1.9 ± 0.2 after DTT treatment, Table 1). There is also no significant difference between the mechanosensitivity of MscL A110−112C and wild type channel (Table 1). Together, these data demonstrate that the conductance of the MscL channel can be specifically decreased by the generation of disulfide bridges within the TM2/CB linker and then restored on disulfide cleavage.

Bundling the TM2/CB Linker by Engineering a Heavy Metal Binding Site within It Allows for the Reversible Decrease in Channel Conductance. We have previously demonstrated that MscL channel activity can be modulated by heavy metals subsequent to engineering a heavy metal binding site within the pore.[31] Therefore, it seemed possible that a similar approach may allow a reversible alteration in MscL conductance. Toward this end, we generated two MscL histidine mutants: A110H and A112H. The presence of Zn^{2+} could theoretically coordinate the histidine residues within the complex, and therefore lead to what was effectively cross-linking at the specific sites mutated. All point histogram analyses showed that the single channel currents of MscL A110H and A112H were 74.9 ± 1.7 and 80.2 ± 3.6 pA, respectively. $ZnCl_2$ treatment in the bath solution indeed dramatically decreased the single channel current of MscL 110H and 112H to 35.7 ± 2.1 and 43.1 ± 3.9 pA (Table 1), respectively. Interestingly, the open dwell time of the main opening of MscL after $ZnCl_2$ treatment is significantly increased, suggesting the unexpected stabilization of a substate. The effect of $ZnCl_2$ on the conductance of both mutants was reversible after $ZnCl_2$ was washed out. The representative current traces and all point histograms of MscL A110H and A112H are shown in Figure 6. The pressure required to open MscL A110H was unchanged (138.3 ± 13.8 mmHg before $ZnCl_2$ treatment and 133.5 ± 13.1 mmHg after the treatment, Table 1); the pressure for MscL A112H showed only a modest decrease in sensitivity (136.3 ± 14.0 mmHg before $ZnCl_2$ treatment and 150.2 ± 16.9 mmHg after treatment, Table 1). $ZnCl_2$ had no effect in the conductance and pressure threshold of the wild type MscL channel, which is consistent with a previous study.[31] Thus, cross-linking at A110H or A112H by heavy metal coordination could effectively and reversibly decrease the conductance of the MscL channel.

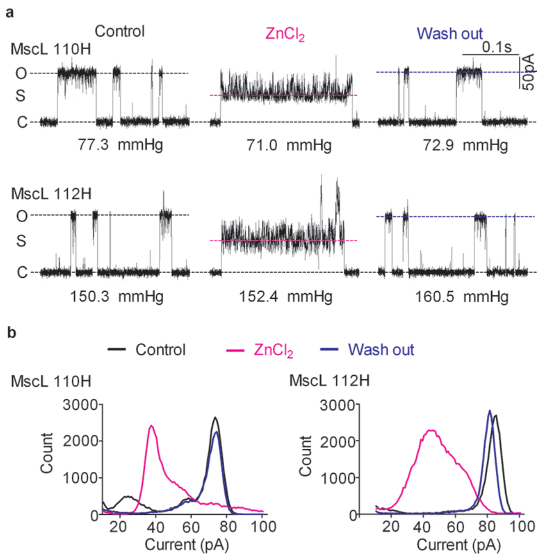

Figure 6. Currents of MscL single histidine mutants (A110H and A112H) are decreased by $ZnCl_2$. (a) Patch clamp recordings of MscL mutants A110H and A112H. "O" and "C" refer to full opening and closing of the channel, respectively, and "S" refers to low level opening or substance of the channel. As shown, the current is decreased by $ZnCl_2$ treatment in the bath solution and is reversed to the control level by washing out the $ZnCl_2$. Pressure applied to patch pipet is shown in mmHg. (B) The all point histogram analyses, reflecting the decrease in conductance of MscL A110H and A112H by $ZnCl_2$ treatment.

Implications for Nanotech-Devices. There is growing interest in the use of biosensors in nanotechnology. For such purposes, these results add to the argument that the MscL nanovalve is extremely pliable. Previous studies have shown the modality of the MscL sensor can be changed to light or pH.[19,20] In addition, the permeation of specific charged molecules can be adjusted.[32] Here we demonstrate that conductance and likely the pore size can also be modified *via* three different methods. Modifying MscL modality and permeation as well as adjusting the pore permeation has implications in the versatility of the MscL nanovalve in nanodevices. For example, one group has proposed using MscL as a triggered nanovalve in drug-release devices.[20] The wild type MscL channel has a huge pore size of greater than 30 Å, which allows facile translocation of both large and small compounds. Modifying the length of the cytoplasmic linker would allow one to now engineer a MscL nanovalve with a throttled pore that will only pass smaller molecules. Thus, if two engineered MscL nanovalves responsive to different stimuli are used within a single vesicular-based nanodevice, stimulus-dependent differential release of drugs or other payloads through the nanovalve could be contemplated. Other groups propose using ion channels as molecular switches in tethered lipid bilayers on a chip surface;[33,34] indeed, MscL has been reconstituted and shown to function in such a microchip device.[22]

In this system, the ability to reversibly decrease the channel conductance may be of advantage. One could imagine modulating the conductance of MscL A110−112C by changes in redox conditions. Such modulation could be useful in "resetting" the gain of a microchip device that has reached saturation. However, the variability in the type and extent of cross-linking at C110−112 may limit this approach. On the other hand, Zn coordination of 110H or 112H leads to controlled, reversible, and characteristic changes in channel behavior with the primary conductance being reduced by half. In summary, this contribution illustrates three additional routes to extend the flexibility in engineering a MscL nanovalve with a set of desired properties, including a variable pore size. The range of MscL modification makes these channels more versatile for use in an assortment of nanodevices.

CONCLUSIONS

We have demonstrated that the CB is quite stable, even upon channel opening. This finding suggested that the MscL nanovalve can be engineered to have a smaller pore size and conductance by decreasing the TM2/CB linker, which appears to serve as a molecular sieve. The linker can easily be modified in at least three ways: shortening by deletions, or constraining the region by either cross-linking or heavy metal coordination; in the later two cases the decreases in pore size are even reversible. These results provide routes for engineering MscL sensors that are more versatile for potential nanotech devices.

METHODS

Strains and Cell Growth. The *E. coli* MscL mutants were generated using the Mega Primer method as described previously.[35] Mutants were inserted within the pB10b or pB10d expression construct, a modified pB10b plasmid.[27,36,37] *E. coli* strain PB104 (Δ*mscL:Cm*)[36] was used for *in vivo* assays and electrophysiological analysis. Cultures were routinely grown in Lennox Broth (LB) medium (Fisher Scientific, Fair Lawn, NY, USA) plus ampicillin (100 μg/mL) in a shaker-incubator at 37 °C and rotated at 250 cycles/min. Expression was induced by the addition of 1 mM isopropyl-β-*d*-thiogalactopyranoside (IPTG) (Anatrace Inc., Maumee, OH, USA).

***In Vivo* Disulfide Trapping.** Overnight cultures were diluted 1:100 and grown 1 h at 37 °C in LB. 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 in LB media) or shocked in water (with or without 15 μM Cu-phenanthroline) at a 1:20 dilution for 20 min at 37 °C. Samples were pelleted at 4000*g* 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.[30,38] The primary antibody anti-Penta His (Qiagen, Valencia, CA, USA) was used at 1:2000 and the secondary Goat anti-Mouse HRP (Bio-Rad, Hercules, CA, USA) at 1:40000. Blots were developed using HRP substrate (Millipore, Billerica, MA, USA) and exposed to film.

Electrophysiology. *E. coli* giant spheroplasts were generated and used in patch-clamp experiments as described previously.[38,39] Excised, inside-out patches were examined at room temperature under a membrane potential of −20 mV. Patch buffers used are 200 mM KCl buffer composed of 200 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$, and 5 mM HEPES (Sigma, St. Louis, MO). The pH of patch buffer was 6.0, except for MscL A110H and A112H, which need to be treated with $ZnCl_2$ at pH 8.0 for histidine coordination. $ZnCl_2$ at 0.5 μM to 2 mM was applied to bath solution for 5 to 20 min for this purpose. 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, USA). A piezoelectric pressure transducer (World Precision Instruments, Sarasota, FL, USA) was used to monitor the pressure introduced to the patch membrane by suction throughout the experiments. Where possible, the pressure thresholds required for MscL gating within single patches were compared before and after treatment (e.g., addition and washout of Zn^{2+}). To compare MscL pressure threshold sensitivities between patches of native membranes, MscS was used as an internal standard as described previously;[27,36] the changes in mechanosensitivity of MscL were measured by comparison of the pMscL/pMscS pressure threshold ratios. Measurements were performed using Clampfit9 from Pclamp9 (Axon Instruments, Union City, CA, USA). Agarose (Bio-Rad Hercules, CA, USA) bridge (2% agarose in 200 mM KCl buffer) was used as the reference electrode.

To cleave disulfide bridges formed in the cysteine mutant MscL channels, 2−10 mM Dithiothreitol (DTT) was applied in to the bath solution for 3 to 20 min. As previously described,[7,15,32] 2−4 mM $MTSET^+$ (2-(trimethylammonium)ethyl methanethiosulfonate bromide) (Toronto Research Chemicals Inc., North York, Canada) was applied in bath solution (Cytoplasmic side of MscL) and single spontaneous opening of MscL channel with G22C or G22C plus Δ110−5 was achieved by opening a single channel *via* suction to allow access of $MTSET^+$.

Calcein Efflux Assay. The calcein efflux assay was performed as previously described.[19] Pet21a plasmid was used for the construction and hosted in strain PB116, which is a λDE3 lysogenization (Novagen, San Diego, CA, USA) of PB106.[27] The MscL proteins were tagged with a multihistidine tag at their c-terminal ends. The histidine-tagged MscL proteins were purified[10] and reconstituted into lipid vesicles composed of (in molar) 70% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 20% cholesterol and 10% 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (Avanti Polar Lipids Inc., Alabaster, AL, USA). After protein reconstitution was performed at 60 °C for 30 min in the presence of Anapoe-X-100 (Anatrace Inc.), calcein was added and allowed to equilibrate with the liposomes at room temperature for 20 min. The detergent was removed by biobeads incubation overnight at 4 °C. Free calcein was then removed by passage through a G-50 fine Sephadex column (GE Healthcare Inc., Piscataway, NJ, USA) washed with vesicle buffer (10 mM Tris-HCl, 500 mM sucrose, pH 8.0). Vesicles were placed into clear 96-well plates and their fluorescence was recorded at 538 nm with the excitation at 485 nm using a Fluoroskan Ascent (Thermo Scientific Inc., Waltham, MA, USA). The baseline of samples was recorded for 5 min followed by 30 min recording in the presence of 1 mM $MTSET^+$. Vesicles were finally lysed by the addition of 0.5% Triton X-100 to determine the total fluorescence levels of calcein encapsulated in vesicles.

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data; P.B. and L.Y. wrote the manuscript with contributions from all other authors; P.B. coordinated and oversaw the project. The authors claim no conflict of interest.

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