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Global structural changes of an ion channel during its gating are followed by ion mobility mass spectrometry

Albert Konijnenberg^{a,1}, Duygu Yilmaz^{b,1}, Helgi I. Ingólfsson^{b,c}, Anna Dimitrova^b, Siewert J. Marrink^{b,c}, Zhuolun Li^d, Catherine Vénien-Bryan^d, Frank Sobott^{a,e,2}, and Armağan Koçer^{f,2}

^aBiomolecular & Analytical Mass Spectrometry Group and ^eUA-VITO Center for Proteomics (CFP-CEPROMA), University of Antwerp, 2020 Antwerp, Belgium;

^bDepartment of Biochemistry and ^cZernike Institute for Advanced Materials, University of Groningen, 9747 AG, Groningen, The Netherlands; ^dInstitut de Minéralogie, de Physique des Matériaux et de Cosmochimie, Sorbonne Universités, CNRS UMR 7590, Université Pierre et Marie Curie, 75005 Paris, France; and ^fDepartment of Neuroscience, University of Groningen, University Medical Center Groningen, 9713 AV, Groningen, The Netherlands

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Mechanosensitive ion channels are sensors probing membrane tension in all species; despite their importance and vital role in many cell functions, their gating mechanism remains to be elucidated. Here, we determined the conditions for releasing intact mechanosensitive channel of large conductance (MscL) proteins from their detergents in the gas phase using native ion mobility–mass spectrometry (IM-MS). By using IM-MS, we could detect the native mass of MscL from *Escherichia coli*, determine various global structural changes during its gating by measuring the rotationally averaged collision cross-sections, and show that it can function in the absence of a lipid bilayer. We could detect global conformational changes during MscL gating as small as 3%. Our findings will allow studying native structure of many other membrane proteins.

ion mobility mass spectrometry | MscL | membrane proteins | structure function | ion channel gating

One of the best candidates to explore the gating of mechanosensitive channels is the mechanosensitive channel of large conductance (MscL) from *Escherichia coli*. The crystal structure of MscL in its closed/nearly closed state from *Mycobacterium tuberculosis* revealed this channel as a homopentamer (1). Each subunit has a cytoplasmic N- and C-terminal domain as well as two α -helical transmembrane (TM) domains, TM1 and TM2, which are connected by a periplasmic loop. The five TM1 helices form the pore and the more peripheral TM2 helices interact with the lipid bilayer.

MscL detects changes in membrane tension invoked by a hypoosmotic shock and couples the tension sensing directly to large conformational changes (1, 2). On the basis of a large body of structural and theoretical data, numerous gating models of MscL have been proposed (3–9). These models agree upon (i) the hydrophobic pore constriction of the channel and (ii) the channel opens by an iris-like rotation—i.e., a tilting and outward movement of transmembrane helices that make the channel wider and shorter (5). This mechanism is supported by patch-clamp (10), disulfide cross-linking (11), FRET spectroscopy (12), and site-directed spin labeling EPR experiments (6, 7), as well as computational studies (13–15). So far, direct experimental results have only been observed for short-range local structural changes, and no measure of the overall global structural changes during channel gating have been reported. Because there is no crystal structure available for the open MscL channel, elucidating overall global structural changes from the onset of channel activation is of utmost importance for our understanding of the gating mechanism of mechanosensitive channels. Here, we provide direct experimental evidence for the key areal changes occurring during channel gating by combining our ability to activate MscL in a controlled manner to different subopen states (16) with a native ion mobility–mass spectrometry (IM-MS) approach.

Results and Discussion

Detergents that are Unstable in the Gas Phase Allow Detection of MscL in Its Native Form. Native mass spectrometry (MS) relies on the gentle ionization and transfer of intact complexes from solution into the gas phase by means of nanoelectrospray ionization (nanoESI) using desolvation voltages and pressures inside the mass spectrometer, which maintain noncovalent interactions (17–21). Together with ion mobility, an adjunct technique that measures collision cross-sections and determines the global (rotationally averaged) size of individual particles, native MS is increasingly used to determine the subunit composition, stoichiometry, size, and shape of biomolecular complexes (22–25). IM-MS has proven exceptionally useful for providing insights into the structure of membrane proteins, such as subunit stoichiometries and phospholipid binding (26, 27). Current methodology depends strongly on the apparently protective capabilities of detergent micelles to transfer membrane proteins intact into the vacuum of the mass spectrometer (26, 28), followed by the removal of these detergents by collisional activation. However, the energy required to rid the membrane protein of its detergent cover often results in the loss of structural integrity (28, 29). To enable analysis of native MscL structure using IM-MS (22–25), we addressed this problem and screened several detergents for their ability to preserve and

Significance

Understanding the working mechanism of membrane proteins is difficult even when crystal structures are available. One promising approach is ion mobility–mass spectrometry (IM-MS) that detects not only the mass-to-charge ratio but also the area of proteins by measuring the rotationally averaged collision cross-sections (CCS) in the gas phase. We identified detergents that allow the release of membrane proteins at low levels of collisional activation for native MS, thus avoiding denaturing effects. We studied the gating mechanism of an ion channel, which occurs through large conformational changes. Ability to detect several coexisting states during gating with a change as small as 3% will open new avenues for studying dynamic structures of membrane proteins.

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¹A. Konijnenberg and D.Y. contributed equally to this work.

²To whom correspondence may be addressed. Email: a.kocer@umcg.nl or frank.sobott@uantwerpen.be.

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release oligomeric MscL, hence eliminating the need for collisional activation.

First, we introduced the protein in *n*-dodecyl- β -D-maltoside (DDM) micelles using nanoESI into the mass spectrometer. DDM is one of the most commonly used and better-studied detergents for native MS of membrane proteins (30). The dissociation of DDM micelles in the gas phase requires moderate to high levels of collisional activation, i.e., 150–200 V. When using IM-MS to study MscL in DDM, we could only detect monomeric MscL, indicating that these conditions were too harsh to keep native oligomeric MscL intact (Fig. 1*A* and Table S1). Next, we tested Triton X-100. Surprisingly, we found that rather than requiring collisional activation to disassemble detergent–protein complexes, the protein already appears in the spectra at very mild declustering conditions as a series of pentamer peaks centered around 4,750 *m/z* (Fig. 1*B*). It appears that Triton X-100 micelles dissociate readily in the gas phase of the mass spectrometer, but we nonetheless detected pentameric MscL with only a few remaining detergent molecules still attached (Fig. 1*B*, Lower). Upon increasing the collision energy above 50 V to remove these remaining detergents, we found that the energy required to do so was high enough to partially unfold MscL in the gas phase (Fig. S1). We therefore kept the various accelerating voltages inside the mass spectrometer below the threshold for unfolding, accepting broader peaks due to the remaining Triton X-100 detergent molecules but avoiding any artifactual structural change of the protein complex.

We hypothesize the ease of protein release from Triton X-100 micelles in the gas phase by their greatly reduced hydrogen bond-forming capacity. Detergent micelles form in water largely due to the hydrophobic effect, which is absent in the vacuum of the mass spectrometer. For the maltoside-based detergent DDM, a network of hydrogen bonds in the head group stabilizes the

detergent clusters in the gas phase, which then would require considerable additional energy for dissociation. Triton X-100, however, is not able to form such an extensive hydrogen bond network because of its polyethylene oxide head group; its micelles disassemble spontaneously upon transfer into the gas phase, thus revealing the embedded oligomeric protein. To examine this hypothesis, we also tested *N,N*-dimethyldodecylamine-*N*-oxide (LDAO), which was not expected to form extensive hydrogen bond networks. LDAO indeed required only 10 V of collision energy to release the protein from its detergent micelles, but yielded only monomeric and dimeric MscL subunits (Fig. 1*C*). This observation highlights the fact that though LDAO is a suitable detergent for gentle protein release in the gas phase, it does not enable MscL to adopt the correct (oligomeric) form in micelles (31, 32). Our approach, using detergent micelles that are unstable in the gas phase, allowed for the detection of native pentamer of *E. coli* MscL by IM-MS. Recently, a study on determining the specificity of membrane proteins toward lipids reported that a homologous MscL channel from *Mycobacterium tuberculosis* (Tb-MscL) also keeps its folded conformation in the gas phase of IM-MS (33). MscL from these two organisms differ in their molecular weight, tension sensitivity, and lipid interactions. Tb-MscL is bigger than *E. coli* MscL. Indeed, in the study of Laganowsky et al. (33), the molecular mass and collision cross-section (CCS) of *M. tuberculosis* MscL was measured as 85,578 Da and 5,438.5 Å², whereas we measured the same parameters for WT *E. coli* MscL as 78,342 Da and 4,861 Å² (Table S2).

Ion Mobility-Mass Spectrometry Can Distinguish Individual Heteropentameric MscL Channels by Their Mass-to-Charge Ratio and Size.

Now able to detect pentameric MscL by IM-MS, we set out to follow a range of large global structural changes occurring during MscL gating, i.e., the opening or closing of the channel. In its closed state, the pore diameter is ~3.5 Å (1). However, when the channel fully opens, the pore diameter is estimated to become as large as ~30 Å (34, 35), which can be detected by IM-MS if it were possible to trigger MscL opening in the absence of the lipid bilayer and tension. Here, to open these tension-sensitive channels in our experimental setup, we used heteropentameric MscLs comprised of varying number of WT and cysteine point-mutated subunits (G22C) per pentamer (Fig. 2) (16). Even though in nature, MscL opens in response to tension in the lipid bilayer, attaching charged cysteine-specific compounds to the pore of MscL-G22C homopentamers opens the channel spontaneously (36–38). Recently, based on this principle, we gained control on the degree of MscL opening in the absence of tension. We increased the hydrophilicity of the channel pore one subunit at a time using WT G22C heteropentameric MscLs. As the number of G22C subunits per heteropentamer increased, hence also the number of binding sites for positive charges within the pore constriction, the channels opened further and visited higher subconducting states (16).

We first tested any conformational differences in individual heteropentameric channels in their closed form using IM-MS. We generated homo- (WT₅ and G22C₅) and heteropentameric (WT₄G22C₁, WT₃G22C₂, WT₂G22C₃) channels using a duet expression system, as described previously (16). Briefly, two *mscL* genes (WT-MscL with a StrepII-tag and G22C-MscL with a 6His-tag) were cloned and coexpressed in *E. coli* PB104, which allowed the production of homo- and heteropentameric channel assemblies in the host cell membrane. Individual heteropentamers were then obtained by using a three-step purification protocol; two-step affinity chromatography to remove the homopentameric MscL, followed by chromatofocusing to separate the individual heteropentameric MscL based on their isoelectric points (Fig. S2). Using IM-MS we determined the mass-to-charge ratio and the rotationally averaged size (CCS) of individual pentamers (Table S2). MscL had a narrow charge distribution (15–20+). In native MS, the lowest-observed significant charge state is known to represent

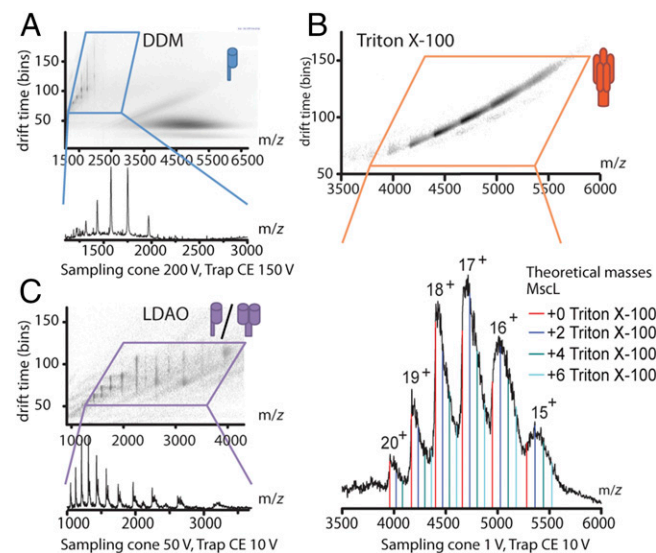


Fig. 1. Comparison of the oligomeric states and collisional activation required to release MscL from detergent micelles in the gas phase. (A) Ion mobility spectrum (Upper) and the excerpt of the mass spectrum in the region where monomeric MscL is observed when released from DDM micelles. Release of MscL from DDM micelles requires high collision energies, which are already enough to disrupt the MscL pentamer, yielding only monomeric subunits. (B) Ion mobility spectrum (Upper) and complementary mass spectrum (Lower) of pentameric MscL released from Triton X-100 micelles. Colored lines indicate calculated *m/z* values for pentameric MscL with and without several detergent molecules attached (average mass 625 Da for Triton X-100 used). (C) Ion mobility spectrum (Upper) and the excerpt of the mass spectrum in the region where monomeric and dimeric MscL is observed when released from LDAO micelles.

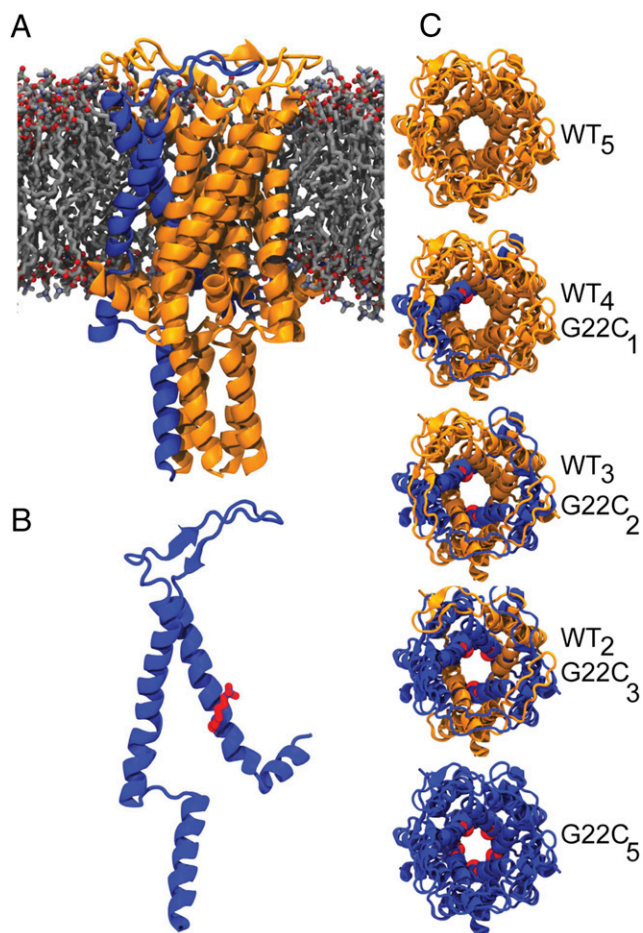


Fig. 2. Structure of MscL. (A) MscL viewed along the membrane plane. (B) MscL monomer highlighting the MTSET bound to the cysteine at position 22 (red). (C) Top view of homo- and heteropentameric MscL with different numbers of WT (yellow) and G22C (blue) subunits. MscL figures were aligned to the *E. coli* sequence and are based on the crystal structure of pentameric WT MscL from *M. tuberculosis* (40).

the native conformation best. We chose the 16+ state for CCS determination throughout, because it gave consistent signal quality for all samples. The CCS was found to increase slightly with the number of WT subunits within a given heteropentamer. This observation was attributed to the larger size of the StrepII-tag of WT monomers relative to the size of the 6His-tag. Indeed, IM-MS of the homopentameric WT MscL channels with StrepII-tag gave a larger CCS than the same protein with 6His-tag (Table S2).

MscL Gating Can Be Detected Reversibly by Ion Mobility-Mass Spectrometry. To see whether the gating of MscL can be detected by IM-MS, we first showed the covalent binding of a positively charged cysteine-specific channel activator [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET) to cysteines of G22C subunits by tandem MS (Fig. S3). We tested increasing amounts of MTSET with G22C₅ homopentamer and showed that MTSET concentrations above 500 μ M maximally label the cysteines (beyond 1,000- μ M deterioration of the IM-MS signal occurs due to ion suppression). Therefore, for the rest of the experiments, we used 750 μ M MTSET for activating MscL channels.

Next, we measured the rotationally averaged collision cross-section of G22C₅ homopentamer in the presence of 750 μ M MTSET. These MTSET activated homopentamers generated similar narrow charge-state distribution (15–20+) as the closed channels, and we followed the 16+ charge state throughout.

Though the closed channel had a single CCS centered around $4,960 \pm 63 \text{ \AA}^2$ (CCS \pm range), after MTSET treatment, the CCS of G22C₅ increased and showed four distinct peaks at $5,095 \pm 46$; $5,330 \pm 48$; $5,580 \pm 56$; and $5,890 \pm 106 \text{ \AA}^2$ (CCS \pm range), suggesting various subopen conformations of the channel during its gating (Table S2). To prove that the presence of MTSET had opened the channel, we closed these already activated channels by using a reducing agent, DTT, for cleaving MTSET off the protein and showed that the CCS returned back to its original value (Fig. 3A). We also tested the effect of MTSET alone and showed that it did not cause any changes in the CCS of WT₅ homopentamer. To further demonstrate that the CCS species of MTSET-activated MscL are different open conformations of the channel, we compared them to collision-induced unfolded MscL (Fig. S1), which shows different states. To our knowledge, this is the first time that ion channel gating is observed by using IM-MS to monitor global conformational changes.

The Overall Size of MscL Channels Increases as the Number of Charges in the Pore Increases. Finally, we could resolve the channel opening to different subopen states by activating heteropentameric MscL with varying numbers of chargeable G22C subunits in the presence

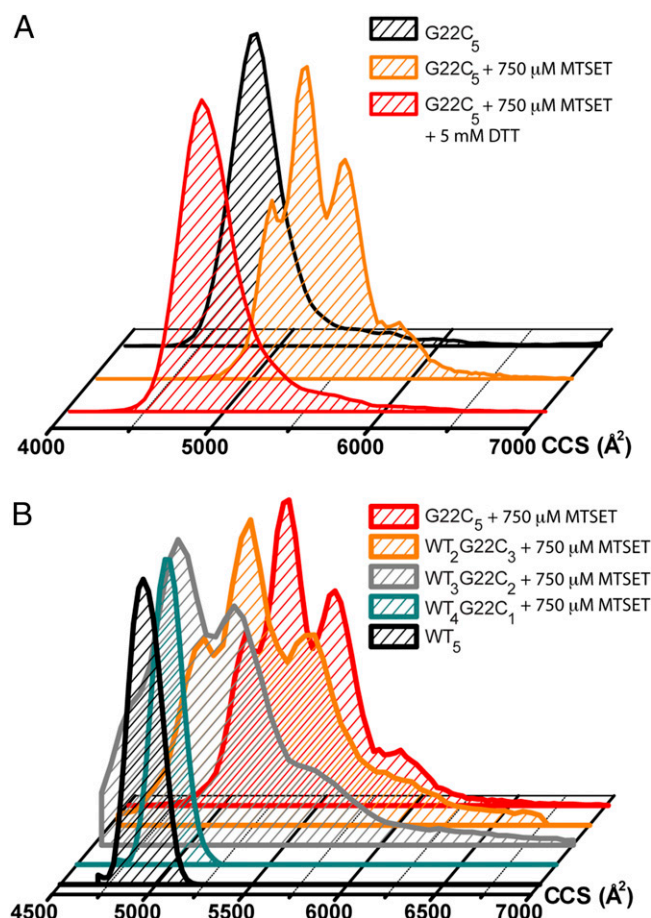


Fig. 3. Opening of MscL observed by IM-MS. (A) Charge-induced opening of MscL is reversible. The shift and dispersity of the collision cross-section observed for the 16+ charge state of G22C₅ MscL (black) upon binding of MTSET indicates opening of the channel (yellow). After adding DTT to the same sample, thus removing the positively charged MTSET from the pore lining, the channel returns to its closed state again (red). (B) Increasing the number of MTSET-labeled cysteines in the pore lining allows MscL to occupy more extended conformations. Reported collision cross-sections are corrected for the different compositions of the purification tags of the heteropentamers of MscL.

resonance (EPR) spectroscopy and transmission electron microscopy. Together with site-directed spin labeling, EPR provides information on protein structure and dynamics both in detergent and lipid bilayers (44). To make MscL visible for EPR, we partially (50% spin-labeling efficiency) labeled homopentameric G22C mutant with 1-oxyl-2,2,5,5-tetramethylpyrrolin-3-yl)methyl methane thiosulfonate. G22 position of MscL has been previously shown to change its environment when the channel opens (6, 7). We monitored structural changes of MscL in Triton X-100 by following the variations in spin–spin interactions, and the mobility of the spin labels within MscL as a function of MTSET activation of the channels (Fig. S5). If the channel opens, the distance between the spin labels increases and causes a change in the EPR line shape, and an increase in the mobility of the spin labels. The overall line broadening in the absence of MTSET indicated closer proximity between spin labels (i.e., stronger spin–spin interaction) in the closed form (Fig. S5, black trace). However, upon MTSET treatment, broadening disappeared (Fig. S5, red trace), suggesting that the subunits of MscL moved further apart. Furthermore, interaction with MTSET increased the probe mobility (Fig. S5).

Next, we used transmission electron microscopy and image analysis to study the closed (no MTSET) and partially open (750 μ M MTSET) states of G22C₅ MscL in Triton X-100 detergent micelles. Negatively stained closed and partially open MscL channels appear as bright spots as shown in Fig. S6A, *Left* and *Right*, respectively. Using WEB and SPIDER software packages, 1,403 closed and 1,004 open-state, centered, and aligned channel images were averaged. In its closed form, the area of the averaged MscL images in detergent was 5,007 \AA^2 (Fig. S6B, *Left*). Upon treatment with MTSET, the channels became flatter and wider and expanded to 6,165 \AA^2 (Fig. S6B, *Right*). Even though we took the averaged images as a measure, we were able to classify open and closed channels into different subclasses using the WEB and SPIDER software (Fig. S6 C–F). Whereas Fig. S6 C and D shows the three classes in the closed form of MscL from the top and the side, respectively, Fig. S6 E and F show three classes of the open state from the top and side, respectively. The observed subclasses may represent different subclosed and subopen states; however, it needs further investigation. Furthermore, the EM data on the averaged images of the closed and partially open state with a $27 \pm 5\%$ area increase upon MTSET opening is in good agreement with the CCS increase found by IM-MS of $21 \pm 3\%$. Together, EM data strongly supports that our observation with IM-MS shows MscL gating in the absence of detergent or membrane.

In conclusion, detergent micelles that require minimum energy for their disassembly in the gas phase are valuable tools in native IM-MS of integral membrane protein assemblies. Resolving the global shape of the protein in combination with its mass-to-charge ratio made it possible to follow the global structural changes of MscL upon its gating. We could detect conformational changes as small as 3% in the rotationally averaged collision cross-sections of the protein complex from the closed (4,952 \AA^2) to the first detectable substate of 5,095 \AA^2 , which seem to represent a closed-expanded state at the initiation of MscL gating. Assignment of other CCS species to defined subconducting states of MscL as defined by patch clamp on the basis of ionic conductance is currently difficult. Because a minor increase in a subconducting state, and hence change in pore diameter does not necessarily make a detectable change in the overall conformation of the channel. Indeed, in patch-clamp studies (39) we could detect as many as seven subconducting states of G22C₅ when activated by MTSET. However, we could observe four CCS species in IM-MS.

We believe studying membrane proteins in gas phase unstable detergents by employing IM-MS opens new avenues for further discoveries on their dynamic structures.

Methods

Sample Preparation and Native IM-MS. MscL homo- and heteropentamers were produced as described elsewhere (16) and introduced into the mass spectrometer (SYNAPT G2; Waters), which was carefully tuned for transmission of noncovalent complexes using nanoESI. Homo- and heteropentamer samples were incubated for 5 min at room temperature with 750 μ M of MTSET before analysis.

MD Simulations. Simulations of MscL gating were performed using the Martini coarse-grain (CG) model (45–47) and the GROMACS 4.X simulation package (48) following a similar protocol as described previously (14, 15). In short, the topology of MscL was derived from the crystal structure of the closed state Tb-MscL (PDB ID code 2OAR) (1, 40) using CG Martini 2.0. The channel was solvated in 562 CG DOPC lipids and $\sim 20,000$ CG water beads (corresponding to $\sim 80,000$ water molecules) using the *insane.py* script. The temperature and pressure were controlled using the Berendsen thermostat (298 K) and barostat (49). The initial system was energy-minimized (steepest descent, 500 steps) and simulated for 1 ns using short time-step 1- to 10-fs simulations and with position restraints on the protein backbone. The restraints were released, the time step set to 30 fs, and the system was equilibrated for 4 μ s with 1 bar semisotropic pressure coupling. Bilayer tension was incrementally applied in seven short (3-ns) simulations to a value of 65 dyn/cm and then simulated for 0.6 μ s. In the first ~ 100 ns following the application of the lateral bilayer tension and thinning of the bilayer, the MscL transmembrane helices tilted, extending the extracellular cavity of the channel. The channel hydrophobic gate takes an additional ~ 200 ns before expanding and opening the channel. Selected frames from the CG simulation were backmapped into atomistic coordinates using the Wassenaar et al. (50) reverse-transformation method and minimized with the GROMOS 54a7 force field (51). The minimum pore radius was measured for the same backmapped frames using the HOLE 2.0 program (52). The radius was defined as the radius of the largest sphere that could pass through the channel hydrophobic lock (~ 0.5 – 1 nm along the channel pore axes starting from the center of mass of residue 18) and interacting with the fully atomistic structure using their van der Waals radii.

All CCS calculations were done using MOBCAL (53, 54) with the projection approximation (PA) algorithm at 298 K and He as a buffer gas. To correct for the use of N₂ in the IM-MS experiments and the underestimation of cross-sections by the PA algorithm, all calculated CCS were scaled by the experimentally derived factor of 1.14 (55). Simulations snapshots were generated using the molecular graphics viewer VMD (56).

EPR Spectroscopy. Continuous-wave EPR measurements were performed using a commercially available MiniScope benchtop X-band EPR spectrometer (MS400 Magnetech GmbH) with a rectangular TE102 resonator. The microwave power was set to 10 mW and the B-field modulation amplitude to 0.20 mT. EPR glass capillaries (0.9 mm inner diameter) were filled with sample volume of 50 μ L with a final protein concentration of 285 μ M. The microwave and the modulation frequencies were 9.41 GHz and 100 kHz, respectively. Each spectrum corresponds to the accumulation of 36 scans.

Electron Microscopy. The 0.03-mg/mL MscL in 100 mM ammonium acetate pH 6.8 buffer was applied in its closed or partially open state, i.e., activated by MTSET, to electron microscope grids and stained with 2% uranyl acetate. The specimen was analyzed with a JEOL 2100, LaB₆ operating at 200 kV. A Gatan CCD camera was used (4,000 \times 4,000; pixel size 15 \AA) for collecting the data. Micrographs were recorded at a nominal magnification of 40,000 (pixel size 2.65 \AA). The WEB and SPIDER software packages (57) were used for image processing. The 1,403 particles in the closed state and 1,004 particles in the partially open states were picked by hand, windowed, subjected to reference free alignment, and sorted into classes using the K-means clustering (58). Each class contained images of particles viewed from the same orientation.

Chromatofocusing. Heteropentamers of MscL were separated by chromatofocusing based on their surface pI as described previously (16). The 250- μ L fractions were collected and their pH determined at 6 $^{\circ}$ C. Only the peak fractions were used for efflux and IM-MS experiments.

Fluorescence Dequenching Assay. Proteins were reconstituted into synthetic liposomes in the presence of a self-quenching fluorescent dye according to Koçer et al. (59). The resulting proteoliposomes were tested for the MscL activity using a fluorescent dequenching assay (59). The data were fitted to the exponential plot, and the first-order rate constants (k) for the fluorescent dye efflux were calculated for each heteropentamer.

The experimental procedures are described in more detail in *SI Methods*.

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