Chemically Charging the Pore Constriction Opens the Mechanosensitive Channel MscL

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ABSTRACT MscL is a bacterial mechanosensitive channel that protects the cell from osmotic downshock. We have previously shown that substitution of a residue that resides within the channel pore constriction, MscL's Gly-22, with all other 19 amino acids affects channel gating according to the hydrophobicity of the substitution (K. Yoshimura, A. Batiza, M. Schroeder, P. Blount, and C. Kung, 1999, *Biophys. J.* 77:1960–1972). Here, we first make a mild substitution, G22C, and then attach methanethiosulfonate (MTS) reagents to the cysteine under patch clamp. Binding MTS reagents that are positively charged ([2-(trimethylammonium)ethyl] methanethiosulfonate and 2-aminoethyl methanethiosulfonate) or negatively charged (sodium (2-sulfonatoethyl)methanethiosulfonate) causes MscL to gate spontaneously, even when no tension is applied. In contrast, the polar 2-hydroxyethyl methanethiosulfonate halves the threshold, and the hydrophobic methyl methanethiolsulfonate increases the threshold. These observations indicate that residue 22 is in a hydrophobic environment before gating and in a hydrophilic environment during opening to a substate, a finding consistent with our previous study. In addition, we have found that cysteine 22 is accessible to reagents from the cytoplasmic side only when the channel is opened whereas it is accessible from the periplasmic side even in the closed state. These results support the view that exposure of hydrophobic surfaces to a hydrophilic environment during channel opening serves as the barrier to gating.

INTRODUCTION

Mechanosensitive channels are ubiquitous in plants, animals, fungi, and bacteria, contributing to proprioception, hearing, kidney, and vascular mechanics in animals (for reviews see French, 1992; Bargmann, 1994; Sackin, 1995; Hamill and McBride, 1996; Kernan, 1997; Sukharev et al., 1997; Sachs and Morris, 1998). Although at least three mechanosensitive activities (MscL, MscS, and MscM: mechanosensitive channel conductance large, small, and mini) are present in *Escherichia coli* (Sukharev et al., 1993; Berrier et al., 1996), two proteins, MscL (Sukharev et al., 1994) and YggB (Levina et al., 1999) are known to contribute to MscL and MscS channel activities, which respond to hypotonic shock (Blount et al., 1997; Ajouz et al., 1998; Levina et al., 1999; Nakamura et al., 1999; Berrier et al., 2000.)

Expression of the 15-kDa MscL, which has only two transmembrane helices (TM1 and TM2), is necessary and sufficient for MscL mechanosensitivity and solute permeation (Sukharev et al., 1994). As seen in the crystal structure of the closed MscL homolog of *Mycobacterium tuberculosis* (Tb-MscL) at 3.5-Å resolution, five TM1s line the pore. Toward the cytoplasmic end these form the presumed channel gate, a hydrophobic bowl closed off both above and below (Chang et al., 1998; Sukharev et al., 1999a). Therefore, MscL's TM1 and TM2, which are closely packed in the closed state, must undergo extensive conformational changes during gating (channel opening) to produce a hole big enough to conduct 3 nS, i.e., an opening some 30–40 Å in diameter (Cruickshank et al., 1997; Sukharev et al., 1999b).

Previously we changed Gly-22 of *E. coli* MscL (Eco-MscL) into all other 19 amino acids (Yoshimura et al., 1999). A gain-of-function screen (Ou et al., 1998), which highlighted cells that die upon expression of mutant *mscLs*, underscored this and other residues along one face of the lower half of TM1. By homology to the Tb-MscL structure, Gly-22 of Eco-MscL resides close to the periplasmic end of the channel pore constriction, which ranges from residues 16 to 23. Mutating residue 22 generated changes in channel gating threshold and kinetics dependent upon the hydrophobicity of the novel amino acid (Yoshimura et al., 1999). In general, hydrophilic substitutions resulted in easy gating channels and cell death, whereas hydrophobic changes made channels harder to open and were well tolerated during normal growth. The substitutions revealed that residue 22 is stabilized in a hydrophobic environment in the closed state and encounters a hydrophilic environment during channel opening to a substate. This is reasonable, because MscL gating is modeled to require all 10 helices to serve as barrel staves forming the channel lumen (Cruickshank et al., 1997; Chang et al., 1998; Sukharev et al., 1999b). This requires an energetically costly move, the exposure of previously hidden hydrophobic surfaces to water (Batiza et al., 1999), a barrier which stabilizes the closed state in the absence of stretch.

In the present study, we test the environment of G22C MscL by using various methanethiosulfonate (MTS) reagents to manipulate the hydrophobicity at residue 22. In

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contrast to our previous work, this experimental design requires only one mutation (G22C), which has no dramatic effect on growth, although it makes the channel harder to open (Yoshimura et al., 1999). Because there are no other cysteines in the entire 136 residues of wild-type MscL, this single change provides a unique sulfhydryl group on each of the identical five subunits within the channel. Therefore, MTS reagents, which vary in length and hydrophobicity and are specifically reactive to sulfhydryl groups, can be added to chemically modify these groups if accessible. As initially described by Akabas et al. (1992) and as reviewed in Karlin and Akabas (1998), such reagents have been successfully used for almost a decade to analyze the state-dependent accessibility of introduced cysteine residues (for example, Pascual et al., 1995; Yang and Horn, 1995; Yang et al., 1996; Larrson et al., 1996; Liu et al., 1997; Pascual and Karlin, 1998; Wilson and Karlin, 1998; Zhang and Karlin, 1998). MTS reagents were also used to estimate the electrostatic potential in the acetylcholine receptor (Pascual and Karlin, 1998) and the size of the inner pore of Kir2.1 channels (Lu et al., 1999).

This method is used here to probe a residue's local hydrophobicity (or hydrophilicity) as well as accessibility. We found that gating exposes residue 22 to the cytoplasmic solution containing MTS reagents. In contrast, this residue is not accessible to the cytoplasm when the gate is closed. Whereas a hydrophobic moiety at this position makes the channel harder to open, a hydrophilic addition at this position helps to overcome the mechanical work required to open the channel to an intermediate substate, a result consistent with our previous study (Yoshimura et al., 1999). In addition, we found that G22C is accessible to reagent from the periplasmic side even in the closed state. These results make it possible to chemically modify purified G22C MscL in an effort to crystallize open MscL channels.

MATERIALS AND METHODS

Wild-type (Sukharev et al., 1994) or G22C (Yoshimura et al., 1999) *mscL* was expressed in the *mscL*-knockout *E. coli* strain PB104 (Blount et al., 1996) using the vector pB10b (Ou et al., 1998). Mutant strains missing *KefA* (MJF453) or *YggB* (MJF455) in addition to *MscL* were gifts of I. Booth (Levina et al., 1999).

Giant spheroplasts were prepared as in Blount et al. (1999). In brief, cells were grown into log-phase in modified Luria-Bertani medium (0.5% NaCl instead of 1% NaCl in the standard LB medium; Martinac et al., 1987) and treated with 0.06 mg/ml cephalexin. At the end of 1.5 h of growth, the bacteria were incubated in the presence of IPTG (isopropyl-β-D-thiogalactoside) for 5 min (wild type) or 15 min (G22C) and then collected by centrifugation and digested with lysozyme (0.2 mg/ml). Pipettes with a resistance of 3.4–4.1 MΩ were used for patch clamp. All experiments were carried out on inside-out patches held at 20 mV (pipette positive) except for experiments in which [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET) was added to the bath before catching the spheroplast with the pipette. In this case, current was recorded in the cell-attached configuration. Currents were amplified and filtered at 5 kHz (Axon 200B, Foster City, CA). Data were stored with a computer using pClamp6 software (Axon). Suction applied through the pipette was monitored with a pressure gauge (XFPM-100KPGV, Fujikura, Tokyo, Japan). The pipette solution contained 200 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$, and 5 mM HEPES (pH 6.0); the bath was the pipette solution with 0.3 M sucrose added. MTSET, 2-aminoethyl methanethiosulfonate hydrobromide (MTSEA), sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES), 2-hydroxyethyl methanethiosulfonate (MTSEH), and methyl methanethiolsulfonate (MMTS) were from Toronto Research Chemicals (North York, Ontario, Canada). The MTS reagents were freshly dissolved, kept on ice, and used within 1 h. The final concentration of each MTS reagent (1 mM MTSET, 2.5 mM MTSEA, 10 mM MTSES, 20 mM MTSEH, and 20 mM MMTS) was determined by its reactivity (Stauffer and Karlin, 1994; Pascual and Karlin, 1998). Dithiothreitol (DTT) was from Sigma Chemical Co. (St. Louis, MO).

A series of suction tests, each lasting several seconds, was applied to the same patch before and after various bath perfusions. The suction needed to open G22C MscL varied from 190 to 380 mm Hg among patches of different geometry. Therefore, the ratio of the suction needed to open a MscL channel relative to that required to open wild-type MscS, which is invariably present in the same patch, was expressed as the MscL gating threshold, as in previous studies (Blount et al., 1996; Yoshimura et al., 1999). Because the suction required to gate MscS decreased slightly after the application of charged MTS reagents to the open channel (see Fig. 5), we used only the suction required to open MscS before MTS application for comparison in each patch.

RESULTS

Reaction of G22C MscL with MTSET

The membrane from a spheroplast of *E. coli* cells expressing G22C MscL was excised with a patch pipette in the inside-out configuration, and the patch membrane was stretched by applying negative pressure through the pipette. Low suction opened MscS mechanosensitive channels with a small unit amplitude (~25 pA at 20 mV; Fig. 1 *A*, *i*, triangle), whereas a higher suction opened MscL channels with a larger unit amplitude (~75 pA; Fig. 1 *A*, *i*, arrow). The gating threshold, or ratio of the suction required to gate G22C MscL relative to that required to gate MscS (see Materials and Methods), was 2.02 $\pm$ 0.22 (n = 25). This value was consistent with the gating threshold previously reported, 2.21 $\pm$ 0.23, and was significantly higher than that of the wild-type MscL (1.64 $\pm$ 0.08, Yoshimura et al., 1999).

After the inside-out patch was formed and 1 mM MTSET, a positively charged MTS reagent, was subsequently introduced to the bath solution by perfusion, gradually increasing suction was applied to the patch membrane. In the presence of MTSET, G22C MscL started opening at a pressure similar to, although only slightly lower than, that before application (Fig. 1 *A*, *ii*). However, upon opening, the channel behavior changed drastically. Unlike in the absence of MTSET (Fig. 1 *A*, *i*), the conductance trace indicates the channels became flickery and would only partially close. As the suction was gradually released, the behavior of the channels changed again. The channels continued to be flickery but closed to a greater and more variable extent. Strikingly, they remained flickery, even in the absence of suction. When suction was applied for a second time, the current, still flickery, again increased with the pressure (Fig.

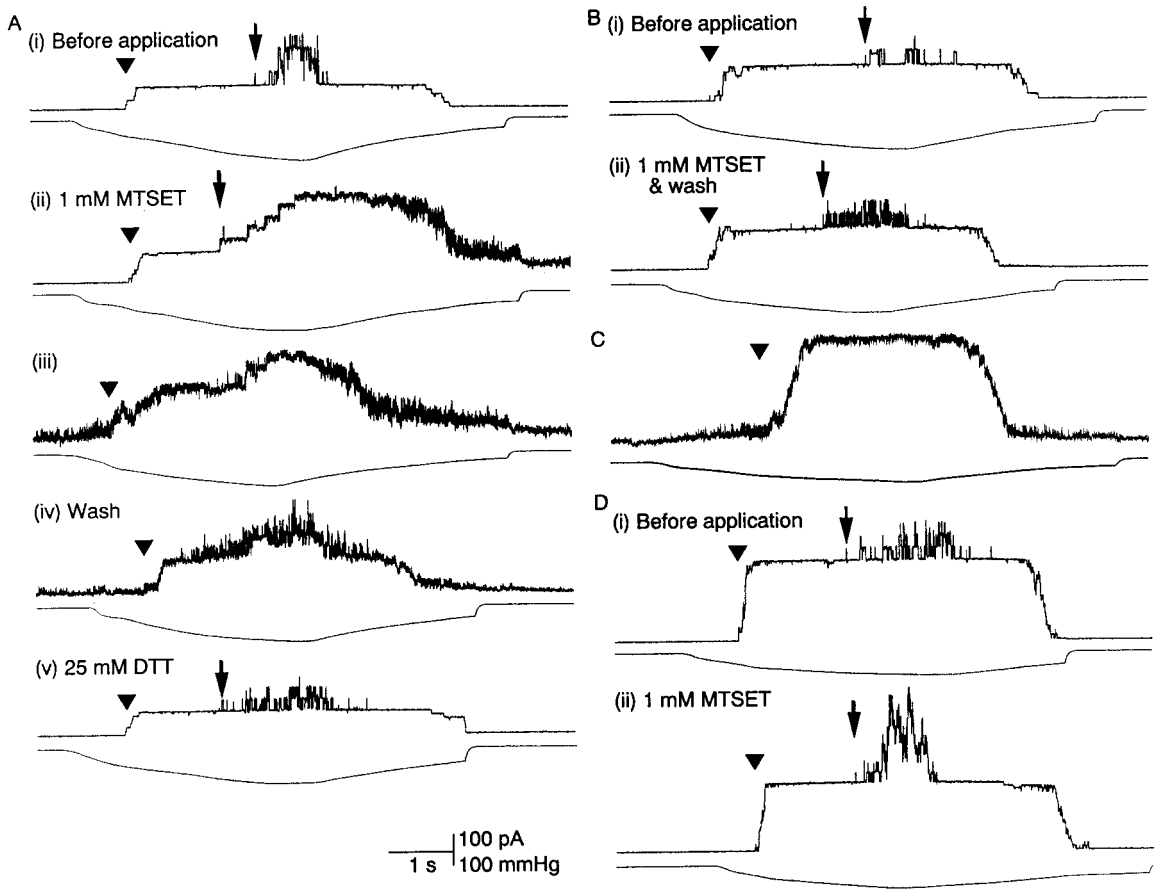

FIGURE 1 Responses of G22C MscL in patches excised from giant spheroplasts when 1 mM MTSET was applied. Each recording shows the current (*upper trace*) during an episode of suction application (*lower trace*). The arrowhead and the arrow indicate the first opening of MscS and MscL, respectively. (*A*) The records from a single patch in the absence of MTSET (*i*), the first test after the application of 1 mM MTSET (*ii*), the second test (*iii*), after washing out the MTSET by perfusion (*iv*), and after the application of 25 mM DTT (*v*). (*B*) The response from a second patch of G22C before the application of MTSET (*i*) and after the application and retrieval of MTSET in the absence of pressure (*ii*). (*C*) Cell-attached recording of the spheroplast that had been incubated in 1 mM MTSET. (*D*) The response to pressure from wild type before the application of MTSET (*i*) and after the application of MTSET (*ii*) (using a treatment similar to that in *A iii*) and while suction is applied.

1 *A*, *iii*). Even after MTSET was washed out, the channels opened spontaneously and maintained their flickery appearance (Fig. 1 *A*, *iv*).

If the changes described above were due to the reaction with MTSET through a disulfide bond, they might be reversed by treatment with a reducing agent, such as DTT. We therefore applied 25 mM DTT to the bath solution of channels treated with MTSET and found that the spontaneous openings disappeared (Fig. 1 *A*, *v*). G22C MscL could now open fully without flickers, and the gating threshold approached that before the application of MTSET. Therefore, the stable fully open state was regained.

In the first trial just after the application of MTSET (Fig. 1 *A*, *ii*), the current trace appeared almost normal until MscL opened. However, in the following trials (Fig. 1 *A*, *iii* and *iv*), this first part of the trace was already dramatically altered, even before suction was applied. This suggests that MscL does not substantially react with MTSET until the channel is first opened. To test this further, we applied MTSET to the bath for 5 min and washed it away without applying suction. After this protocol, the gating threshold was similar to that before any MTSET treatment (Fig. 1 *B*). Threshold measurements from a number of spheroplasts showed slight, but significant changes in the gating threshold after this apply-and-wash protocol and during the first trial in the presence of MTSET (Fig. 2 *A*). There were also subtle changes in channel kinetics after the apply-and-wash protocol. However, we did not explore this effect further because this change was not consistently observed with the other MTS reagents (Fig. 2 *A*, see below). Nonetheless, despite such minor effects, the large decrease in the gating threshold indicated by spontaneous openings clearly occurred only after the channels were opened in the presence of MTSET applied from the cytoplasmic side.

Therefore, these observations suggest that G22C MscL in its closed state does not react rapidly with MTSET pre-

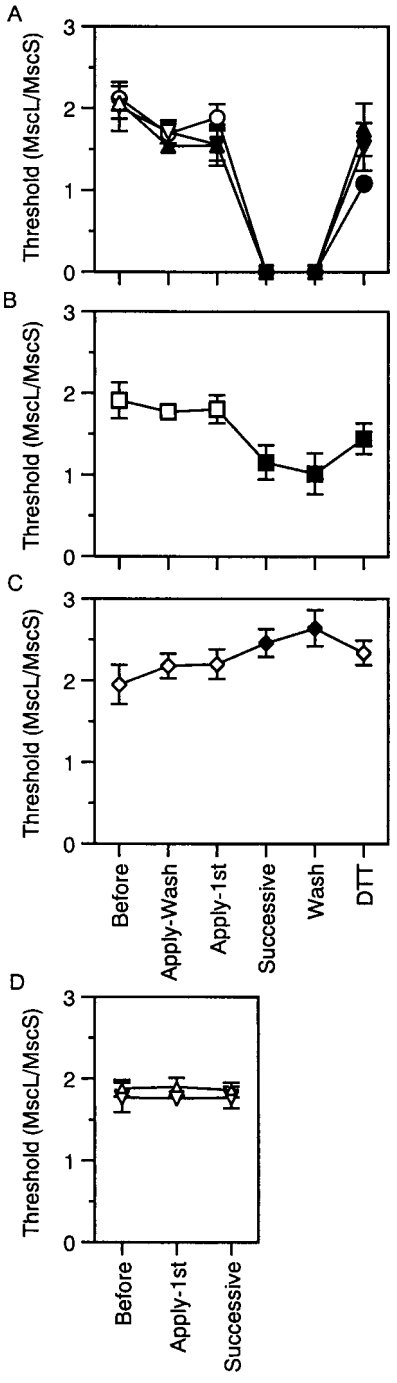

FIGURE 2 The changes in the gating threshold of G22C MscL (*A–C*) and wild-type MscL (*D*) caused by MTS reagents. The threshold of the pressure to open MscL is expressed as the ratio to that required to open MscS before application of MTS reagents. The MTS reagents used are as follows: (*A*) hydrophilic MTSET (△ and ▲, $n = 7$), MTSEA (▽ and ▼, $n = 6$), and MTSES (○ and ●, $n = 4$); (*B*) the polar MTSEH (□ and ■, $n = 5$); (*C*) the hydrophobic MMTS (◇ and ◆, $n = 3$). Shown is the threshold (mean ± SD) before an MTS reagent was applied (Before), after an MTS reagent was applied but washed away without applying any suction (Apply-Wash), when an MTS reagent was applied and suction was applied for the first time (Apply-1st), when suction was applied in succession (Successive), after the bath MTS reagent was perfused away (Wash), and when 25 mM DTT was applied after wash (DTT). The data that are statistically different ($p < 0.05$) from the threshold before the application of the MTS reagents are shown by filled symbols.

sented from the bath (cytoplasmic) side of the excised patch. The findings that 1) the G22C MscL threshold did not change substantially before the channel was opened in the presence of MTSET and that 2) the threshold decreased to zero once the channel had reacted with MTSET have been consistently observed in all of a number of patches. The G22C MscL gating threshold difference before and after the MTSET reaction is statistically highly significant (Table 1). This effect was removed by adding DTT but not by simple bath perfusion (Figs. 1 *A*, *v*, and 2 *A*).

In addition to the change in G22C MscL, we found that the threshold of MscS also decreased slightly on application of MTSET but to a lesser extent (12.0 ± 3.7%; see Fig. 5). Because the threshold of MscS changed by application of MTSET and other MTS reagents, the gating threshold of MscS before application of MTS reagents was used to normalize the threshold of MscL throughout the following experimental steps.

Because G22C was inaccessible to MTSET from the intracellular side in the closed state, we also tested MTSET applied to the bath (periplasmic side) before capturing cells in the pipette. We found that flickery channel activity was present, even as the patch was formed by applying weak negative pressure (lower than the threshold of MscS). This activity continued even after the pressure was released. The current trace after periplasmic exposure to MTSET with subsequent patch formation and then application and release of additional pressure (Fig. 1 *C*) was similar to the successive trial (Fig. 1 *A*, *iii*) after MTSET had been applied from the cytoplasmic side.

Reaction of G22C MscL with MTSEA, MTSES, MTSEH, and MMTS

MTSET bears a positively charged amino group near the end of an aliphatic chain. To examine how the charge and hydrophilicity of MTS reagents may affect the gating of G22C MscL, we applied a different positively charged MTS reagent (MTSEA), a negatively charged MTS reagent (MTSES), a polar MTS reagent (MTSEH), and a hydrophobic MTS reagent (MMTS). These were applied to the cytoplasmic side of inside-out patches (Figs. 2 and 3 and Table 1). The concentration of each reagent was adjusted to compensate for the reaction rate (see Materials and Methods).

When other charged MTS reagents (2.5 mM MTSEA or 10 mM MTSES) were applied, G22C MscL behaved in essentially the same manner as in the experiment with MTSET applied to the cytoplasmic side. There was little decrease in threshold when suction was applied for the first time in the presence of these charged MTS reagents or when

TABLE 1 Mechanical sensitivity of MTS-bound G22C MscL and G22X MscL

MTS-bound G22C MscL				G22X MscL*		
MTS	R†	Threshold‡	n§	Amino acid	Side chain	Threshold‡
None		2.02 ± 0.22	25	Cys		2.21 ± 0.23
MTSET	$CH_2CH_2N^+(CH_3)_3$	0.0 ± 0.0	7			
MTSEA	$CH_2CH_2NH_3^+$	0.0 ± 0.0	6	Lys	$CH_2CH_2CH_2CH_2NH_3^+$	0.23 ± 0.27
MTSES	$CH_2CH_2SO_3^-$	0.0 ± 0.0	4	Glu	$CH_2CH_2CO_2^-$	0.10 ± 0.18
MTSEH	CH_2CH_2OH	1.12 ± 0.12	5	Ser	CH_2OH	1.14 ± 0.14
MMTS	CH_3	2.55 ± 0.24	3	Ala	CH_3	2.47 ± 0.20

*Data from Yoshimura et al. (1999). Gly 22 was replaced with the amino acid shown.
†R of MTS reagents, CH_3SO_2SR. MTS binds to cysteine in a configuration of Cys-S-S-R.
‡Threshold expressed by the ratio of the pressure required to open MscL and MscS.
§Number of spheroplasts examined. The threshold was determined for three to five times in each spheroplast.

the MTS reagent was applied and washed out without any stimulation (Fig. 2 *A*). Under these conditions, a statistically significant decrease ($p < 0.05$) was not detected, except for a small decrease by MTSEA in the first trial. Once the channels had been opened in the presence of MTSEA or MTSES, they opened spontaneously in the absence of pressure, and their flickery activities increased with the pressure (Fig. 3, *A* and *B*, and Table 1). The initial threshold was restored in part when 25 mM DTT was applied (Fig. 2 *A*).

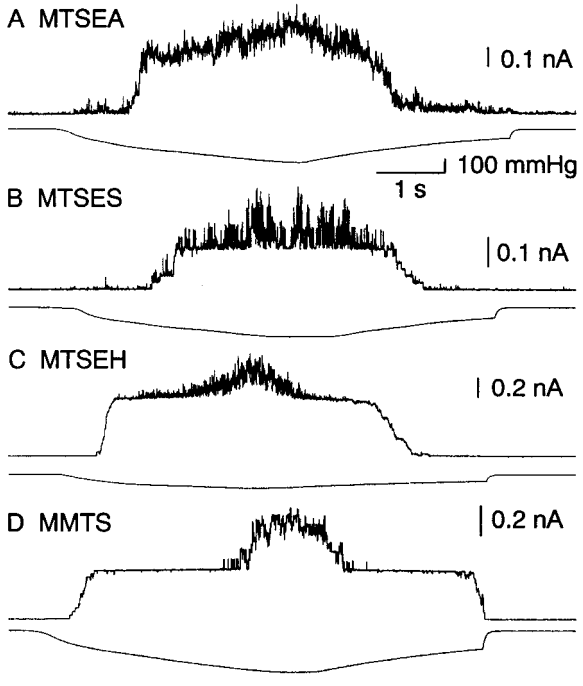

FIGURE 3 Ensemble behavior of G22C MscL in excised inside-out patches after bathing in the positively charged MTSEA (*A*), negatively charged MTSES (*B*), polar MTSEH (*C*), and nonpolar MMTS (*D*). The MscL channels had been opened in the presence of the MTS reagent before this experiment; thus, the experimental step corresponds to that of Fig. 1 *A*, *iii*. Each record is typical of several episodes from a number of patches, consisting of a current (*upper*) and a pressure (*lower*) trace.

In contrast to the dramatic reduction in the G22C MscL gating threshold after the channel had previously been opened in the presence of charged MTS reagents, the threshold decreased by only 50% after a similar treatment with the polar MTSEH (Fig. 2 *B* and Table 1). Because this threshold is close to that of MscS, both channels began opening almost simultaneously when suction was increased (Fig. 3 *C*). In contrast, the threshold increased by 25–35% when 20 mM of the hydrophobic MMTS was applied in a similar way (Figs. 2 *C* and 3 *D* and Table 1). This value is close to the pressure at which the membrane lyses (~2.5 times the MscS threshold).

Single-channel openings of G22C MscL bound to MTS reagents

Besides the gating threshold, hydrophilic MTS reagents affected the mode of the channel openings. As is shown in Fig. 4, *A–D*, the G22C MscL reacted with hydrophilic MTS reagents showed a pronounced substate of ~5–20 pA (at 20mV) in addition to the flickery openings into the fully open state of 75 pA, especially at low suction. The amplitude of the most stable substate was 6.6 ± 3.8 pA in MTSET, 18.0 ± 2.6 pA in MTSEA, 11.0 ± 1.0 pA in MTSES, and 11.5 ± 4.9 pA in MTSEH. Such substates were rarely observed in untreated G22C MscL (Fig. 4 *E*). Unlike the hydrophilic MTS reagents, the hydrophobic MMTS did not induce the substate opening (Fig. 4 *F*).

Although G22C MscL reacted with MTSET, MTSEA, MTSES, and MTSEH preferred the open substate, it did not dwell long in the fully open state (<1 ms) (Fig. 4, *A–D*). The openings were flickery up to the pressure at which unmodified G22C MscL was activated (Fig. 3, *A–C*). This contrasts with the opening of untreated G22C MscL, whose channel open-time distribution can be well fitted with two time constants of 14.3 ± 0.3 ms and 2.7 ± 0.8 ms (Fig. 4 *E*). On the other hand, the fully open dwell time of MMTS-G22C MscL appeared to be similar to the control G22C MscL or even longer (compare the typical traces in Fig. 4,

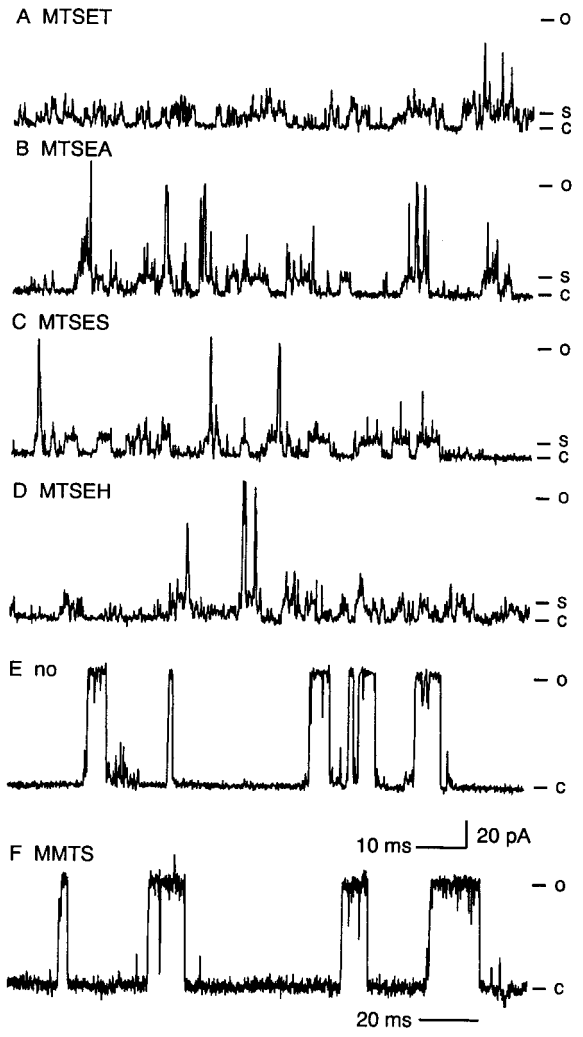

FIGURE 4 Single-conductance activities of G22C MscL showing differences in fully open versus substate distribution with various MTS reagents. The channels had been opened in the presence of the MTS reagent before this experiment. The reagent and the applied pressure (in parentheses; relative to the threshold of MscS) areas follows: (*A*) MTSET (1.12); (*B*) MTSEA (1.03); (*C*) MTSES (1.01); (*D*) MTSEH (1.15); (*E*) without treatment (1.51); and (*F*) MMTS (2.00). The scale bar in *E* applies to *A–E*. The letters c, s, and o mark the unitary current levels in the closed, sub-, and fully open state of the channel.

E and *F*). Quantitative analysis based on a number of channel events was not performed because the membrane easily lysed during prolonged exposure to the high pressure needed to activate MMTS-G22C MscL.

Although the wild-type MscL channel is not ion selective, it is possible that G22C MscL modified with a charged MTS reagent gains some ion selectivity in the open substate. However, we did not observe a significant shift in the reversal potential (less than 5 mV) when solution containing 400 mM KCl was perfused in place of 200 mM KCl in the normal bath solution. Therefore, if such selectivity exists, it is very small.

Effects of MTS reagents on wild-type MscL and MscS activities

Although wild-type MscL contains no cysteines (Sukharev et al., 1994), it is nonetheless possible that certain nonspecific interactions between MTS reagents and MscL might affect MscL's activity. To test this possibility, we examined whether 1 mM MTSET or 2.5 mM MTSEA would affect the mechanosensitivity of wild-type MscL. The channel activities, including the gating thresholds (Figs. 1 *D* and 2 *D*) and channel open times, did not change after wild-type MscL had been opened in the presence of MTSET or MTSEA. This confirms that the changes in the gating of G22C MscL by these MTS reagents are due to the specific binding of the MTS reagents to Cys-22.

As noted above, we observed a 12% decrease in the threshold of MscS when MTSET was applied (Fig. 5). Unlike G22C MscL, the decrease in the threshold of MscS was present from the first trial in the presence of MTSET. The low threshold persisted after the MTSET had been washed out (data not shown). A decrease was also generated in a similar manner when MTSEA and MTSES were applied (Fig. 5). The effects of these MTS reagents were unexpected because YggB, to which the major activities of MscS have been attributed (Levina et al., 1999), also has no cysteine residues in its sequence. Thus, we speculated that the cysteine-bearing KefA, which generates a MscS-like activity (Levina et al., 1999) might contribute to the changes in the MscS traces we registered. To test this idea, we

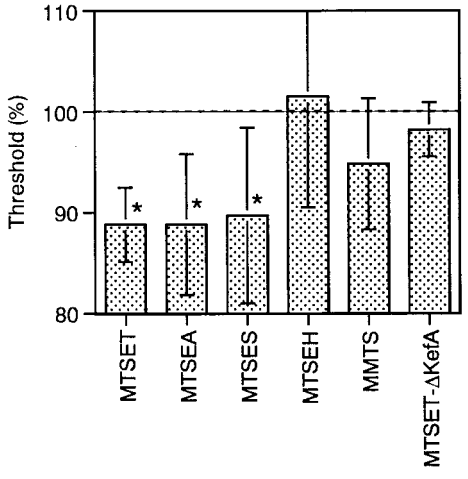

FIGURE 5 Change in MscS threshold after MTS reagent application. The MscS gating threshold in the presence of the newly added MTS reagent (using a treatment similar to that in Fig. 1 A, *ii)* is presented as a percentage of the suction required for gating MscS before any application of the reagent. MTSET-ΔKefA refers to MTSET application to *kefA*$^-$ cells (MJF453); mean ± SD, $n = 5$. When the original pressure data before and after MTS treatment were compared by paired *t*-tests, there were statistically significant differences (*asterisks*) in wild-type MscS treated with MTSEA, MTSET, or MTSES but not in wild-type MscS treated with MTSEH or MMTS or in ΔKefA-MscS treated with MTSET.

applied MTSET to a *kefA*-deficient mutant. The MscS in this mutant activated in a manner that was similar to the wild-type MscS, and the threshold did not decrease after opening MscS in the presence of MTSET (Fig. 5). We have tried to apply MTSET also to a *yggB*-deficient mutant (Levina et al., 1999) but failed because we have not encountered MscS in several trials, consistent with the findings of Levina et al. (1999) that MscS activities are rare in this mutant.

DISCUSSION

MTS reagents induced drastic changes in the activities of G22C MscL. The mechanical gating threshold, the frequency of the substate opening, and the dwell times in the fully open state or substates were all altered by the application of MTS reagents (Figs. 2 and 4). Whereas these changes became apparent only after the channel had been opened in the presence of the MTS reagents applied from the cytoplasmic side (Figs. 1 *A* and 2, *A–C*), application of MTSET from the periplasmic side caused spontaneous opening (Fig. 1 *C*).

The direction and extent of the changes in the gating threshold were determined by the hydrophilicity of the MTS reagents used. For example, a large decrease in the threshold occurred when the charged MTS reagents (MTSET, MTSEA, and MTSES) were applied (Fig. 2 *A*), whereas a moderate decrease was observed when the polar MTS reagent (MTSEH) was applied (Fig. 2 *B*). In contrast, when the hydrophobic MTS reagent (MMTS) was applied, the threshold increased by ~25–35% (Fig. 2 *C*). Thus, the ease of channel gating paralleled the hydrophilicity of the MTS bound to Cys-22.

This trend agrees with our previous result in which the gating threshold of G22X MscL decreased with the hydrophilicity of X, the substituted residue (Table 1) (Yoshimura et al., 1999). For example, MTSEA and lysine, which both bear the positively charged amino group at the end of a hydrocarbon chain, decreased the suction required for opening to zero or close to zero, respectively. (Note that both the disulfide cross-linker and the $-CH_2-$ from cysteine contribute to the side chain when MTS is bound.) MTSES and glutamate, which are negatively charged with a sulfonyl and carboxyl group, respectively, similarly reduced the gating threshold to zero or close to zero. MTSEH and serine, both with a polar hydroxyl group, halved the normal threshold. Finally, MMTS and alanine, which either contribute or have a hydrophobic, methyl-group side chain, increased the threshold (Table 1). In contrast to the comprehensive mutagenesis required in our previous study (Yoshimura et al., 1999), this study manipulates the hydrophobicity of residue 22 by generating only one mutation to which various MTS reagents are attached. Despite the relative simplicity of this method, our results support our previous conclusion that hydrophilicity inside the gate correlates with the mechanosensitivity of MscL.

When a hydrophilic MTS reagent was bound to G22C MscL, the dwell time in the fully open state shortened; this was balanced by long dwells in one of the open substates, giving the conductance trace the appearance of flickers riding on plateaus (Fig. 4). This mode of channel opening was also observed in *E. coli* MscL when Gly-22 was replaced with a hydrophilic amino acid (Yoshimura et al., 1999). These changes can be explained if we assume that residue 22 is in a more hydrophilic environment in the substates than in the fully open state (Yoshimura et al., 1999). One candidate for this hydrophilic environment is the water-filled lumen of the channel from which cytoplasmic MTS reagents can access Cys-22 during gating. Modification of the channel trace when the channel was opening but not when the channel was closed (Fig. 1 *A*, *ii*) supports this view.

The present study shows that the channel needs to be opened before it can be attacked efficiently by MTS reagents presented from the cytoplasmic side but that the channel can be modified simply by exposure to MTS reagents on the cell surface in the absence of suction. Perhaps the Cys-22 side chain creates a cleft below the upper constriction at V23 to allow access, although not preventing closure of the lower constriction at V16, because growth of G22C is relatively normal in the absence of MTS reagents (Yoshimura et al., 1999). Note that a histidine substitution at this position allowed hydrogen access from both above and below and prevented growth (Yoshimura et al., 1999). Alternatively, in contrast to its relative position in the *M. tuberculosis* MscL channel structure (Chang et al., 1998), perhaps residue Gly-22 in the wild-type *E. coli* channel has limited exposure to the periplasm although its overall environment is still hydrophobic.

Although Cys-22 is accessible to water from the periplasmic side, the channel is nonetheless difficult to open in the absence of modification (Fig. 1 *A*, *i*) (Yoshimura et al., 1999). In contrast, the addition of a hydrophilic MTS reagent (Figs. 1 and 2 *A*) or a hydrophilic amino acid (Table 1) (Yoshimura et al., 1999) makes the open configuration more probable, possibly by decreasing the energy difference between the closed and the open state and, thus, the gating threshold. We see the physiological result of this destabilization of the closed state and channel opening: hydrophilic amino acid substitutions kill cells containing these mutant MscLs (Ou et al., 1998; Yoshimura et al., 1999). Therefore, this effect is not due to the accessibility of residue G22C from the periplasmic side, but rather derives from the energy shift created by making residue 22 more hydrophilic. Residue 22 is part of a hydrophobic constriction in the closed state (Chang et al., 1998). However, the preference for an open substate by the introduction of a hydrophilic moiety suggests that residue 22 is in a hydrophilic environment when MscL is in the open substate. Therefore, it seems

reasonable to assume that the work required to expose this largely buried hydrophobic surface to a hydrophilic environment contributes to the energy barrier to gating (Chang et al., 1998; Batiza et al., 1999; Moe et al., 2000). Although G22 is located within this hydrophobic gate chamber, all the buried hydrophobic surfaces exposed during opening, i.e., those residues that contribute to the barrier, are not necessarily within the presumed gate.

Levina et al. (1999) as well as Sukharev and Booth (2000) showed that YggB, which also does not contain cysteines, appears to play the major role in MscS activity observed under a patch. In contrast, deleting only KefA (belonging to the same protein family as YggB, but having five cysteine residues in its sequence) does not seem to affect MscS activity or survival after an osmotic downshock (Levina et al., 1999). It was therefore unexpected to find the threshold of MscS activity also reduced by MTSET, MTSEA, or MTSES (Fig. 5). The absence of this effect in the *kefA*$^-$ mutant (Fig. 5) supports the view that KefA contributes to MscS-like activity (Levina et al., 1999).

Our goal is to crystallize MscL in an open state. However, mutants favoring MscL opening do not grow well and therefore will not supply protein in large quantities. We now know that G22C MscL bacteria grow well (Yoshimura et al., 1999) and that MscL can be encouraged to open by reacting with a charged MTS reagent (this study). Thus we have separated the culturing step from the step altering the channel's gating threshold.

In practice, other technical issues have yet to be faced. Because a stretch force cannot be exerted on purified G22C MscL protein in a detergent solution, one can only rely on the state distribution under no stress. Even in the intact membrane patches, MTS-modified G22C MscL still distributes among several open and closed states (e.g., Fig. 4 *A*). We need to further bias the distribution toward the open states. One way to create permanently open channels might be to engineer additional cysteines into the gate and hope that multiple MTS charges therein further prohibit the closed states.

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