Mechanosensitive membrane channels in bacteria respond to the mechanical stretching of the membrane. They will open when bacteria are subjected to rapid osmotic down shock. MscS is a bacterial mechanosensitive channel of small conductance. It is a heptameric membrane protein whose transmembrane part, including the gate and its kinetics, has been well characterized. MscS has a large cytoplasmic domain of a cage-like shape that changes its conformation upon gating, but its involvement in gating is not understood. We screened MscS for mutations that cause potassium leak in *Escherichia coli* strains deficient in potassium transport systems. We did a phenotypic analysis of single and multiple mutants and recorded the single channel activities of some of them. After these analyses, we attributed the effects of a number of mutations to particular functional states of the channel. Our screen revealed that MscS leaks potassium in a desensitized and in an inactivated state. It also appeared that the lower part of TM3 (transmembrane, pore-forming helix) and the cytoplasmic β domain are tightly packed in the inactivated state but are dissociated in the open state. We attribute the TM3-β interaction to stabilization of the inactivated state in MscS and to the control of tight closure of its membrane pore.

MscS, a bacterial mechanosensitive (MS) channel of small conductance, is one of the best characterized membrane channels. MscS opens when activated directly by membrane stretch, and the same mechanism activates also MscL, another *Escherichia coli* MS channel that has higher threshold and bigger conductance than MscS. When open, both channels will jettison osmolytes protecting the bacteria against severe osmotic down shock (1). MscS is a homo-heptamer, and each subunit consists of three membrane-spanning helices (TM1, TM2, and TM3) and a large cytoplasmic domain (Fig. 1A) (2). Two crystal structures (2, 3) and electron paramagnetic resonance (EPR)-based models of the channel two states, closed and open (4, 5), as well as several molecular dynamics studies (6–13) have been published.

The other structure was solved for the A106V mutant that has been shown to stabilize the open state (14), and it is believed to represent an open or partially open state (3). The structures, experimental data, and modeling studies have led to the models of the closed (12) and open (13) states and pointed toward channel rearrangements upon gating (16–21). The emerging model is that TM3, the channel pore-lining helix, buckles and straightens at glycines 113 and 121. This action happens during transitions between the closed, open, desensitized, and inactivated states (11). Mutation of these glycines to alanines (in G113A/G121A mutant) allows the channel to open but jams it in the open state even after pressure release (11). A few experimental tests have been conducted to support the model; however, the conclusions drawn from them were restricted to the transmembrane part of the channel. The MscS large cytoplasmic chamber was previously shown to change its conformation upon gating (19, 21–23); however, it is not understood nor is it modeled how this cage-like shaped domain is involved in gating. To obtain a comprehensive understanding of MscS gating, an approach that provides a broader search in a model-independent manner is required.

Until now, bacterial MS channels have been functionally characterized in vivo mostly by isolation of so-called "gain-of-function" (GOF) and "loss-of-function" (LOF) mutants. GOF mutants inhibited cell growth because they opened at a lower threshold of activation. On the other hand, LOF mutants were less effective in protecting cells against osmotic down shocks because of impaired opening. Multiple mutants of MscL exhibiting GOF phenotype have been isolated from the random mutant library (24). The isolation was possible by screening for clones that block bacterial growth when overexpressed. This allowed mapping the channel gate even before its crystal structure was known. Similar approaches applied to MscS have been less successful because only one GOF mutant was found (V40D) (25). Analysis of LOF mutants of MscL isolated through random and scanning mutagenesis with mutations in the periplasmic rim of its funnel (26) indicated the import-
Genetic Screen for Potassium Leaky MscS Channels

tance of the lipid-protein interaction. Lipid-protein interactions have been also invoked as crucial in channel function in the study in which externally exposed amino acids from TM1/2 of MscS were mutated to arginines. In this study, few LOF mutants were identified (27).

We used a genetic complementation approach in Escherichia coli strains deficient in potassium transport systems (e.g. LB2003 or TK2446). These strains cannot grow on a media with low (1–10 mM) potassium (28), but expression of potassium channels or transporters can restore their growth (29). This strategy was successful for isolation of potassium channels and transporters (30) as well as the mutations that activate potassium channels (31, 32). A similar approach but utilizing the Saccharomyces cerevisiae K+ transport-deficient mutant was also used to study functional substitutions in the Kir2.1 inwardly rectifying potassium channel (33). The assay also worked for detecting nonphysiological pathways of potassium influx and for isolation of potassium-conducting mutants of transport proteins that are not potassium-selective. In this way single genomic mutants of MscL and ProP were isolated (34), and seven mutants of MscK, a MscS paralog, were tested (35). This was possible due to the high inward potassium electrochemical gradient, so any “leaky” protein could provide a route for potassium influx. This strategy may therefore result in isolation of mutants with a variety of functional changes in protein. We have been able to isolate a plethora of mutations that cause MscS to leak potassium. After phenotypic analysis, we have also been able to assign the role of several of them to the particular functional state of the channel.

Our data presented here indicate the following: (i) the lower part of TM3 (TM3b) and the β domain are tightly packed in the closed and desensitized/inactivated states; (ii) they dissociate upon opening suggesting that MscS leaks potassium in the desensitized/inactivated state, and (iii) this supports the previous proposal that inactivation from the open state involves uncoupling of TM1/2 from TM3 and identifies residues important for the TM1/2-TM3 interaction.

The list of mutants isolated in the screen provides mutant candidates for further detailed studies by means of electrophysiology, EPR, and crystallography.

EXPERIMENTAL PROCEDURES

Media—The K115 media consisted of the following: K2HPO4, 46 mM; KH2PO4, 23 mM; (NH4)2SO4, 8 mM; MgSO4, 0.4 mM; FeSO4, 6 μM; sodium citrate, 1 mM; thiamine hydrochloride, 1 mg/liter; and glucose, 0.2% (w/v) (35). For K0 medium, equimolar sodium phosphate was used to replace potassium phosphate. Suitable mixed proportions of K0 and K115 were used to create a medium with intermediate K+ concentrations. In all media, ampicillin was used at 100 μg/ml, and all plates contained 1.2% (w/v) agarose for potassium-limiting media and 1.5% agar for other media. LB770 broth contained 10 g/liter tryptone, 5 g/liter yeast extract, 170 mM NaCl and was adjusted to pH 7.2. LB770 was the same as LB770 but contained 570 mM NaCl. Measured osmolarities for LB770 and LB770 were 406 and 1198 mosm/kg H2O, respectively.

Strains. E. coli strains deficient in potassium transport systems TK2446 (ΔtrkG(ΔtrkH(ΔtrkA-mscL)) and LB2003 (trkAkup1 (trkD1) kdpABC5 rpsL. metE thi rha gal) were kindly provided by Professor Brad S. Rothberg (Temple University School of Medicine, Philadelphia) and Professor Evert P. Baker (University of Osunbruck, Germany). E. coli strains MJF429(ΔmscK::kan ΔmscS) and MJF465(ΔmscK::kan ΔmscS Δmscl::Cm) were kindly provided by Professor Ian R. Booth (University of Aberdeen, Aberdeen, UK).

Mutant Library Construction and Phenotypic Analysis—MscS random mutant library was constructed using pTRCMscS plasmid (mcs gene cloned into pTRC99A vector). Mutations were introduced with a GeneMorph II EZClone domain mutagenesis kit (Stratagene) according to the manufacturer’s instructions, with the following primers: pTRCrmp, 5′-CGGA-TAACAATTTACCAAGGAAACAG-3′ and pTRCrmlw 5′-AAATCTTCTCTCATCCGAAAACGC-3′. The mutant library was amplified in DH5α strain and transformed into TK2446 and LB2003 strains. Transformants were plated on minimal media containing 1 or 3 mM K+ and 10 or 50 μM IPTG, and the plates were incubated at 37 °C for 2–5 days. Plasmids were isolated from growing colonies and retransformed back to TK2446 or LB2003 for phenotype confirmation. Plasmids were sequenced and mutations identified on a protein level after in silico translation using the standard bacterial codon table.

For site-directed mutagenesis, the QuickChange II site-directed mutagenesis kit (Stratagene) was used according to the manufacturer’s instructions. For GOF phenotype identification, TK2446 or LB2003 cells carrying appropriate plasmids were plated on K115 containing 1 or 2 mM IPTG. The growth was assessed the next day. Clones not growing or growing weakly as compared with the uninduced control were considered to have GOF phenotype. GOF phenotype was also visible in the down shock tests in which the MJF465 strain had been grown for 2.5 h in LB770 medium with 1 mM IPTG followed by plating the cells in serial dilutions as described below. LOF phenotype was determined by down shock survival. Similar assays were described previously (1). In this case, multiple clones carrying appropriate plasmids were grown in a multi-well format. A single colony was used for overnight inoculum into LB770 broth with 100 μg/ml ampicillin, and the culture was grown in 37 °C with moderate shaking (140 rpm). The following morning, 40 μl of the culture was added to 2 aliquots of 760 μl of pre-warmed LB770 broth with 100 μg/ml ampicillin and with (induced culture) or without (uninduced culture) 1 mM IPTG. The cultures were grown in 37 °C with 140 rpm for 2 h and cooled on a table at room temperature for 30 min. Then each culture was diluted 10-fold into LB170 (down shock) or LB570 (the control) and immediately vortexed for 3 s. The cells were serially diluted in LB170 (down shock) or LB570 (control) to a final dilution of 1:270, and 5-μl samples of the final dilution were spotted on LB170 agar plates with 100 μg/ml ampicillin. The plates were left overnight at room temperature with an additional 90-min incubation at 37 °C the following morning. Then the plates were photographed, and the images were analyzed with Corel Photo-Paint X4. In a single experiment, up to 24 mutants were tested. For each set, at least three independent experiments were performed producing similar results.
Electrophysiological Recordings—Single channel recordings were obtained from inside-out excised membrane patches, and the experimental procedure and equipment used were the same as described earlier (19). Bath control solution contained 400 mM sucrose, 200 mM KCl, 90 mM MgCl₂, 4 mM CaCl₂, and 5 mM HEPES, pH 7.2 (18). The pipette solution was the same as the bath solution except it lacked sucrose (18).

Suction, at constant pipette voltage, was applied pneumatically to the patch pipettes using a 10-ml syringe, together with two in-line, three-way valves and was monitored by a pressure manometer PM015D (10.3 kPa or 1.5 p.s.i., WPI, Sarasota, FL). This system allowed us to apply any suction step with an error of <2% of the required level. Intervals of 3 min or longer were maintained between consecutive applications of suction. Data were acquired (with a sampling rate of 5 kHz), filtered at 2 kHz, and analyzed using pCLAMP6 software.

Isolation of E. coli Membranes and Immunoblotting—MscS mutants bearing an N-terminal Strep-tag II were expressed in E. coli. The cells were lysed in PBS supplemented with 1 mM PMSF by sonification for 3 min. Cell debris was removed by centrifugation at 10,000 rpm for 30 min at 4 °C. The membranes were isolated by centrifugation at 100,000 rpm for 30 min at 4 °C. The membranes were resuspended in PBS, and membrane protein concentration was determined in the presence of 1% SDS with BCA protein assay kit (Pierce). Equal amounts of membrane proteins were separated by SDS-PAGE, transferred onto PVDF membranes, and then immunoblotted with antibody against Strep-tag (GenScript Co.). The blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce) for 1 h, and chemiluminescence was detected with an ECL system (GE Healthcare). Structures were visualized using VMD (36) and PyMol programs.

RESULTS

We used a genetic complementation approach in the E. coli strains deficient in potassium transport (TK2446 and LB2003), which cannot grow on a media with low potassium concentration (28). Their growth, however, could be restored by expression of an exogenous channel that allowed for an inwardly directed potassium flow (31). We constructed a random mutant library of MscS in the pTRC99A plasmid. This library was transformed into the E. coli TK2446 and LB2003 strains, and ~10⁷ clones were plated on low potassium. We isolated clones that grew on 1 and 3 mM potassium, with a low concentration of IPTG (10 and 50 μM) that produced a weak induction of protein expression. We opted for weak induction because we expected that some mutants might have a GOF phenotype. These mutants could inhibit growth if overproduced. We realized that there is an optimal level of channel activity required to rescue growth (32), and the level of protein expression should be much lower than that previously used for isolation of GOF mutants (24). Therefore, the screens were performed under low induction such that even potentially toxic mutations could be metabolically tolerated and retrieved. Our expectation was that leaky mutants, meaning channels that complement potassium uptake deficiency, would emerge from the screen.

Catalog of the Mutants and Their Location on the MscS Structure—We isolated 77 unique mutants that grew on low potassium (supplemental Table S1). Among them were five single mutants, and the rest were the multiple ones bearing up to six mutations. Most of the mutations, mainly those with charged and to a lesser extent polar substitutions of hydrophobic amino acids, were found within TM1–3 helices of the channel (Fig. 1, B and C). The list of mutations (supplemental Table S2) clearly shows that mutants with mutations in TM3 were isolated most frequently. While analyzing the results of the screen, the question we are addressing is as follows. How does a single substitution of a particular amino acid contribute to the potassium leak? Because the majority of the mutants had multiple mutations, a straightforward answer would be difficult. In multiple mutants, one or more of the mutations could be responsible for the phenotype, although the others could be neutral. It is possible that multiple mutations...
contributed additively/synergistically to the potassium leak phenotype, or alternatively, the rate of mutagenesis was too high for isolation of single mutants. To narrow down these possibilities and determine which of the mutations is responsible for the phenotype, we selected 4 single and 14 multiple mutants (with 2–5 mutations each) for detailed analysis. We chose multiple mutants in such a way as to include mutations that were scattered over diverse regions of the channel except for mutations in 26 amino acids in the unresolved N-terminal part of MscS. The mutations were studied in single mutants that were isolated in the screen or constructed (Table 1) or as a combination of substitutions in double, triple, and quadruple mutants as found in multiple mutants isolated in the screen (supplemental Table S3). It yielded a set of 66 mutants for further analysis.

Genetic Screen for Potassium Leaky MscS Channels

| II growth on media with K⁺ [mM] | III GOF osmotic downshift survival (LOF) | IV 2mM IPTG | + IPTG |
|---------------------------------|------------------------------------------|-------------|--------|
| Mutant                          |                                          |             |        |
| WT                              |                                          |             |        |
| TM1 L55P                        |                                          |             |        |
| TM2 outer L82E                  |                                          |             |        |
| TM2 inner V65D                  |                                          |             |        |
| TM2 inner F86C                  |                                          |             |        |
| TM2 inner G76D                  |                                          |             |        |
| TM2 inner A85T                  |                                          |             |        |
| TM2 inner L85N                  |                                          |             |        |
| TM3a outer G90S                 |                                          |             |        |
| TM3a outer V96D                 |                                          |             |        |
| TM3a outer V99E                 |                                          |             |        |
| TM3a outer A103V                |                                          |             |        |
| TM3a outer L115Q                |                                          |             |        |
| TM3a outer L116Q                |                                          |             |        |
| TM3a outer A119D                |                                          |             |        |
| TM3a outer M126K                |                                          |             |        |
| TM3b outer A94D                 |                                          |             |        |
| TM3b outer A98S                 |                                          |             |        |
| TM3b outer A1010**              |                                          |             |        |
| TM3b outer G104S                |                                          |             |        |
| TM3b outer A106T                |                                          |             |        |
| TM3b outer L1090**              |                                          |             |        |
| TM3b outer Q112P                |                                          |             |        |
| TM3b outer G113E                |                                          |             |        |
| TM3b outer G113A                |                                          |             |        |
| TM3b outer N117D                |                                          |             |        |
| TM3b outer N117K                |                                          |             |        |
| TM3b outer A125D                |                                          |             |        |
| TM3b outer G121A                |                                          |             |        |
| TM3b outer G121D                |                                          |             |        |
| TM3b beta interface beta domain |                                          |             |        |
| G148E                           |                                          |             |        |
| N167Y                           |                                          |             |        |
| N167I                           |                                          |             |        |

*, due to strong GOF phenotype, no growth during osmotic down shift experiments was observed.

**, due to extreme GOF phenotype, no single mutant could be obtained and analyzed.

TABLE 1
Phenotypic analysis of the single mutant set

The table is also shown as part A in supplemental Table S3 together with part B containing data from the set of multiple mutants. nd indicates not determined.
IPTG was performed to identify mutants with impaired growth in these conditions. Those were recognized as exhibiting a GOF phenotype, which reflects increased channel opening at resting membrane tension. In addition, each mutant was tested in strain MJF465 (ΔmscKNmscLΔmscS) for survival of osmotic down shock. We used two protocols in the tests performed as follows: one under low expression (where there was no exogenous induction, and expression was due to promoter leak) and the other under high expression (induction by 1 mM IPTG). These survival tests enabled us to identify mutations that interfered with gating of the channel and resulted in channels that lost the ability to protect the cell from down shocks (LOF phenotype). Growth and survival tests allowed for precise detection of how a particular mutation may be involved in potassium leak (Table 1, supplemental Table S3, and supplemental Figs. S1 and S2). GOF and LOF phenotypes can give additional information on the role and involvement of particular amino acids during MscS gating. All collected data on the set of 66 mutants are included in supplemental Table S3.

To be clear and concise, we will present and discuss in detail our observations on all 35 single mutants taken from the set of 66 mutants. This group of mutants includes 31 mutants that we constructed and 4 isolated directly in the screen. To gain a better insight into the correlation between location of mutation and its impact on the channel behavior, we mapped the mutations on the MscS high resolution crystal structure (Fig. 2, A–C). We also grouped single mutants according to their associated GOF or LOF phenotype. In addition, we recognized a group of single mutants with “no GOF, no LOF” phenotype, which included mutants with channels leaking potassium but whose growth and osmotic down shock survival remained unchanged.

As detailed below, from the mutants revealed by the screen we analyzed 35 single mutants. Among them there were 7 GOF mutants (3 were described earlier (15, 37)); 12 LOF (5 were found earlier (14, 27)) and 16 no GOF, no LOF mutants (3 were described earlier (11, 15)).

**Genetic Screen for Potassium Leaky MscS Channels**

**Mutants with Substitutions in the Pore-facing Surface of TM3a Exhibit GOF Phenotype**—The screen revealed that polar substitutions of hydrophobic amino acids clustered in the upper pore-exposed part of TM3a resulted in GOF phenotype. These mutations include I97N, A98S, and A101S/A101D (Table 1 and Fig. 2A). The latter one was found only in multiple mutants and resulted in an extremely strong GOF phenotype, which prevented its analysis as a single mutation (supplemental Tables S1–S3). There was an A94D mutation also found in this cluster; however, it did not evoke GOF (Table 1). Serine substitutions of alanines 94 and 98 were introduced previously and shown to lower the threshold of the MscS activation (15). G104S and A106T mutants with mutations in the middle part of the pore-exposed surface of TM3a did not exhibit any obvious growth phenotype. They grew only on a very high potassium concentration (Table 1). The gate mutations (L109P/L109Q) were isolated in multiple mutants; however, we were unable to analyze them as single mutants due to extreme GOF phenotype.

Based on previous findings and the results presented here, we think that, in general, this category of mutations pinpoints regions and residues affecting the channel opening. As a consequence, the channels carrying mutations in the pore region have a lower threshold of opening and a higher probability of opening in vivo than the wild type MscS.

**Mutations at the TM1/2-TM3 Interface Lead to LOF Phenotype**—We found mutants leaking potassium but showing LOF in osmotic shock experiments. The mutations leading to this phenotype were as follows: V65D, A85T, L86N, and G90A on TM2; V96D on TM3a; and L115Q, A119D, and G121D on TM3b (Fig. 2B). A85T does not complement potassium transport deficiency in concentrations up to 15 mM [K⁺] (Table 1) but dramatically enhances potassium leak in the double mutant A85T/G121D (supplemental Table S3).

Interestingly, all but one of these mutations point toward the TM1/TM2-TM3 interface, which is in contrast to GOF mutations that point toward the pore (Fig. 2C). The only LOF-inducing single mutation found beyond this interface was N167Y/N167I localized in the β domain (Fig. 2B). The mutations in TM1/2 and TM3 form two clusters (Fig. 2B). One (upper) cluster is found in the helical turn region around Gly-90, which is a hinge for TM1/2 swinging motion upon the channel transition between open and inactivated states (2). The mutation G90S might decrease hinge flexibility and/or increase hinge angle leading to the detaching of the TM1/2 paddle from the TM3 barrel. This possibility would lead to an idea that the resting conformation of mutated channels may resemble an inactivated state of the wild type channel. The other mutations in this region are V96D, A85T, and L86N. Mutations in the 85 and 86 positions were previously found to result in LOF phenotype, and this was interpreted as an effect of improper lipid-protein interaction (27). Our data presented in this paper and other recently published results (14) suggest that in potassium-leaking mutants, LOF phenotype is rather an effect of broken or loosened protein-protein interactions.

The lower cluster of mutations is located in the crevice between the TM1/2 paddle and the outer (facing the paddle) surface of TM3b (Fig. 2B) and includes V65D on TM2 as well...
as L115Q and A119D on TM3b. These two structures interact in a modeled MscS closed conformation (12) and form a hydrophobic contact between Val-65, Phe-68, and Leu-69 on TM2 and Leu-111 and Leu-115 on TM3 (38). The interaction of the TM1/2 paddle and TM3 is also critical for force transmission from the membrane to the gate and activation of the channel (14). Any perturbation of the contact between interacting residues leads in fact to the inactivated conformation in which the channel is pressure-insensitive (14).

To support the notion that channels with this class of mutations (leaking potassium and showing LOF) reside in vivo in an inactivated state, we chose V65D, L115Q, and A119D mutants for the electrophysiological experiments. We detected channel activity of the V65D mutant in the strain MJF465; however, the number of channels was significantly lower than in the case of the wild type MscS expressed under the same conditions (0–5 V65D channels versus 50–100 wild type channels), and the channel showed fast inactivating, flickery behavior indicating a tendency for closure (supplemental Fig. S3). While testing the L115Q mutant in the MJF465 strain (∆mscK∆mscL∆mscS), we were unable to detect any mechanosensitive channel activity that could be attributed to MscS channels (data not shown). This observation is consistent with a very strong LOF phenotype (Table 1) that may be an effect of a persistent inactivation of the channel. The L115Q mutant, similarly to A119D, was very proficient in potassium leak (growth on 1 mM K⁺), but unlike A119D, it was completely deficient in protection against osmotic down shocks (Table 1). The mutant A119D in TM2 showed very similar activities to those recorded from V65D when expressed in the strain MJF465 (supplemental Fig. S3). To measure a relative threshold of channel activation, A119D was expressed in the strain MJF429 (∆mscK∆mscL). Its activities (if any) overlapped with the activities of MscL whose threshold of activation is significantly higher than that of the wild type MscS (data not shown). This indicates that the mutant channel has a higher activation threshold and points to impaired force transmission between the TM1/2 paddle and the TM3 gate. The level of mechanosensitive activity of the LOF mutants correlates with the severity of LOF phenotype but not with the level of MscS expression. The level of expression of the L115Q mutant is comparable with the level of expression of A119D, but A119D shows channel activity and L115Q does not (supplemental Fig. S4).

The mutants found in this screen confirm recent findings that serine substitutions of the residues in the TM1/2-TM3 crevices (Val-65, Phe-68, Leu-111, and Leu-115) result in channels exhibiting partial LOF phenotype (with L115S giving the strongest LOF phenotype), are inactivated without opening (silent inactivation), and show high thresholds of activation, fast inactivation, and flickery behavior (14). The impact of mutations in this region on potassium leak is not symmetrical; mutants with charged substitutions in TM3 leak potassium better than mutants with charged substitutions in TM2. V65D on TM2 (growth threshold 7 mM K⁺) and A119D on TM3 (growth threshold 1 mM K⁺) can serve as an example (Table 1). Mutations that change hydrophobic residues to polar or charged ones may induce excessive hydration in the hydrophobic crevices between TM3 and TM1/2 and cause detachment of the TM1/2 paddle from TM3. The force transmission from lipid bilayer to the TM3 gate is disturbed in these mutants impeding channel opening and resulting in impaired protection during osmotic down shock (LOF phenotype).

It is known from earlier studies that the process of inactivation of MscS is also determined by a kink in TM3 around Gly-113 (11). Additional glycine at 112 (Q112G substitution) results in a faster rate of inactivation compared with the wild type channel (11). We identified proline substitution of Gln-112, which resulted in LOF phenotype and moderate potassium leak (Table 1). We attribute this to the greater tendency of this mutant to bend around Gly-113 that, in consequence, leads to inactivation providing additional evidence supporting the notion that the channels in inactivated conformation leak potassium.

Mutations in β Domain, a Novel Gating Element—A significant observation emerging from the analysis of mutants, which are revealed in the screen, is that some of them had mutations in TM3b and β domain, respectively. These include the A120D GOF mutant, the N167Y/N167I LOF mutant, and the N117K, G148E, and G168D mutants that have no additional GOF or LOF phenotypes (Fig. 3A). Such positioning and diversity in phenotypes of mutations may indicate that interaction between TM3b and the β domain affects the channel gating. The β domain (amino acids 128–181) with its complicated structure has not yet been included in MscS gating schemes (11–13), and this prompted us to look closer at the mutations in this region.

Especially intriguing is the fact that the single N167Y/N167I mutant isolated in the screen exhibits LOF phenotype similarly to the mutants from the TM1/2-TM3 crevice. The activity of the N167Y mutant channel recorded in the MJF465 strain showed residual, fast inactivating, and flickery channel activity similar to that found in V65D and A119D expressed in the same strain (supplemental Fig. S3). This suggests that the N167Y mutation in the TM3b-β interface, likewise mutations in the crevices between TM1/2 and TM3 (V65D and A119D), enhances channel inactivation. It gave rise to the hypothesis that the β domain plays a role during inactivation.

It was previously demonstrated that the double mutant combining mutations from the TM1/2-TM3 crevice (F68S and L111S) that exhibited partial LOF and silent inactivation, showed strong LOF phenotype with almost permanently nonactive channels (14). We wondered whether a similar phenomenon would be observed if a mutation from TM3-β interface was combined with a mutation from the TM1/2-TM3 crevice. Curiously, such a double mutant combining two mutations that were mentioned before, the V65D substitution in TM2 and N167I in the β domain, was isolated in our screen. The double V65D/N167I mutant had increased potassium leak and also boosted LOF phenotype compared with both the single V65D and N167I mutants indicating concerted action of both mutations (Fig. 3B). Crystal structure (2) reflecting, as it is proposed, the inactivated state of the channel (7) reveals that Asn-167 packs closely with Ala-120 in TM3b. Hydrophobic N167I substitution could then induce a stronger
binding of the β domain to a hydrophobic Ala-120 at TM3b. Based on that, we hypothesized that splaying TM1/2 (because of V65D substitution) and stronger binding of β to TM3b (because of N167I substitution) both act synergistically to induce the inactivated state (Fig. 3C). It seems that in this case channel leak is not due to channel opening but rather due to a leaky inactivated state.

If hydrophobic substitution N167Y/N167I promotes inactivation, mutations of opposite properties in the TM3-β domain interface may result in a channel with impaired (slowed down) inactivation. Two mutations identified in our screen, N117K and G168D, affect polarity of the region. Residues Asn-117 and Gly-168 face each other while being on TM3b and β, respectively. The change of polar amino acid to a positively charged one (N117K) or introducing a negative charge (G168D) results in a higher polarity of the region. Because the TM3b-β domain interface is quite hydrophobic, it is reasonable to think that such mutations will result in a weaker interaction between these two structures.

Patch clamp experiments revealed that both of these mutations resulted in channels that show activity (adaptation to pressure) that is characteristic to the wild type channels (Fig. 4). Both mutations N117K and G168D result in amino acids of opposite charges. Interestingly, combining them into a double mutant N117K/G168D restored the wild type phenotype. The double mutant N117K/G168D shows no potassium leak (data not shown), and its kinetics are similar to the wild type channel (Fig. 4). In single N117K and G168D mutants, unbalanced charges repel TM3b from the β domain. But in the double mutant N117K/G168D, the oppositely charged side chains can form a salt bridge holding both structures together. Apparently such a salt bridge can substitute for native interaction between TM3b and β domain that seems to be needed for adaptation of wild type channels. It therefore appears that molecular compatibility but not the character of individual amino acids forming the TM3b-β domain interface is important for adaptation.

To provide further evidence for that, we introduced cysteine in position 117. The N117C mutant shows kinetics resembling that of the wild type; however, the number of active channels was lower. Cysteine could be modified with methanethiosulfonate reagents by the addition of groups of different properties. We attempted to modify this cysteine with positively charged methanethiosulfonate ethyltrimethylammonium, with negatively charged sulfonatoethylmethane thiosulfonate (MTSES), or hydrophobic methylmethanethiosulfonate (MMTS). Modification with methanethiosulfonate ethyltrimethylammonium resulted in nonfunctional channels (data not shown), which as we think was probably caused by the introduction of a large positively charged group of quaternary amine, which could interfere stronger with the channel function as compared with primary amine group of lysine.
found in the noninactivating N117K mutant. On the other hand, MTSES adds a smaller negatively charged moiety to the side chain of cysteine. MTSES modification resulted in a larger number of open channels and in slower adaptation as compared with the nonmodified control (Fig. 5). The effect of MTSES mimics the effect of mutation introducing opposite charge in this position (N117K). On the other hand, modification of N117C with MMTS introducing the hydrophobic methyl group reduces the number of active channels. All the above effects of methanethiosulfonate reagents could be reversed by the addition of the reducing agent DTT. There was no influence of MTSES or MMTS at 1 mM on wild type channels (data not shown). In our scheme (Fig. 3C), we proposed that interaction of TM3b and the \( \beta \) domain is transient, and they splay apart during opening. The experiments described above did not, however, provide final evidence. To provide it, we constructed the double mutant N117C/G168C. Single N117C and G168C mutants open similarly to the wild type channel with G168C exhibiting slightly slower adaptation (Fig. 6A). Reducing agents do not influence the activity of single cysteine mutants (data not shown). We expressed the double cysteine mutant in the MJF429 (\( \Delta mscK \Delta mscS \)) strain and found that pressures higher than the threshold of MscL did not open the double mutant unless the reducing agent (2.5 mM DTT) was added (Fig. 6). These results indicate that there is a spontaneous formation of S–S bonds in the double mutant under nonreducing conditions, and this locks the channel in a nonconducting, presumably closed state. Reducing S–S bonds allows the channel to open (Fig. 6). The above presented results consistently point to the transient interaction between TM3b and the \( \beta \) domain, which is determined by compatible surfaces on both structures. This interaction is crucial for the desensitization/inactivation process and is involved in tight closure of the channel (Fig. 3C).

**DISCUSSION**

The most noticeable result of our screen is the isolation of mutants with extremely diverse phenotypes. Some of the potassium-leaking mutants we isolated dwell in the open state easily in vitro and exhibit GOF phenotypes in vivo, whereas some open with extreme difficulty in vitro and exhibit LOF phenotypes in vivo. Why is that? Is there any feature common to all potassium-leaking mutants?

The majority of the mutations focus around TM3 pore-forming helices; it therefore seems plausible to think that analyzing them could provide answers to these questions. TM3 helices form a tight hydrophobic gate of the MscS channel, and it has been shown that polar or charged substitutions of hydrophobic amino acids forming the gates of MS channel lower the threshold of channel opening and result in GOF phenotype (37, 39). So it is not surprising that we isolated GOF mutants with substitutions in the pore lumen that leak potassium due to lowering of the opening threshold.

The TM3 helices are surrounded by TM1/2 helices that have been shown to be attached to TM3s in the closed and open states but splay during inactivation of the channel (14,
19). We isolated a number of mutants with mutations in the TM1/2-TM3 crevices. They open with increased threshold or inactivate easily and exhibit correlated LOF phenotype. The mutations are focused in two clusters, the upper and the lower (Fig. 2B).

The lower cluster was also identified in the recent work of Belyy et al. (14). They identified several polar substitutions of hydrophobic amino acids, which result in channels that inactivate without opening (silent inactivation), exhibit partial LOF phenotype, high threshold of activation, fast inactivation, and a flickery behavior. The molecular dynamics studies indicate that polar substitutions mentioned above result in excessive hydration of the crevices; in fact, no water molecules were observed in crevices during molecular dynamics simulation in the wild type channels but some in the F68S mutant (14). The amino acids identified in that study were Val-65, Phe-68, Leu-111, and Leu-115 with the substitution of the last one giving the strongest LOF phenotype. The mutations isolated in our screen overlap in part with those reported by Belyy et al. (14); however, our screen extended the cluster of amino acids by the addition of Ala-119. We also identified a second (upper) cluster of mutations near the Gly-90 hinge.

Channels with mutations in the lower cluster identified in our screen exhibit more severe phenotypes than those reported by Belyy et al. (14). Some of our mutants exhibit no mechanosensitive activity at all (L115Q), and some show activity of a few extremely fast inactivating channels (V65D and A119D). This could be due to the fact that the substitutions in our screen were more drastic than the serine substitutions. It is plausible that our mutants (e.g. V65D or A119D) exhibit a higher degree of hydration of the TM1/2-TM3 crevices as compared with the wild type or the serine mutants. This excessive hydration results in a complete separation of TM1/2 from TM3 forcing the mutant channel to reside in the inactivated state, which is manifested in the LOF phenotype in vivo. Such a phenomenon was previously observed in patch clamp experiments in which the addition of 2,2,2-trifluoroethanol that can partition into hydrophobic crevices in proteins resulted in inactive channels (40) and is consistent with the structure-based “dash-pot” model of MscS inactivation proposed by Akitake et al. (18). In the inactivated state, TM3 helices may exhibit increased mobility around the gate compared with the closed, compact conformation, in which TM1/2 helices restrict the motion of TM3. Increased mobility of TM3...
can result, in turn, in accidental hydration of the gate and ion leak. However, it is also possible that hydration of the crevices could open alternatively to the main gate route for K\textsuperscript{+} ions, analogous to pathway of so called \(\omega\)-current found in mutant Shaker channels (41).

The question arises whether such excessive hydration of TM1/2-TM3 crevices is indispensable for channels to be leaky in the inactivated state. In other words, is the inactivated state of the wild type channel also leaky? One of the mutants repeatedly isolated in our screen was N167Y (or similar N167I) substituting polar to hydrophobic amino acid in the \(\beta\) domain. This mutation results most probably in tighter interaction between the \(\beta\) domain and TM3 because, as revealed by the crystal structure, Asn-167 locates opposite to the hydrophobic Ala-120 on TM3. The fact that our mutants N167Y/N167I exhibit LOF phenotype and fast inactivating, residual activity is consistent with the features of the channels residing preferentially in an inactivated state and suggests that binding of \(\beta\) domain to TM3 may occur during inactivation. Curiously, N167I mutation acts in concert with V65D mutation supporting this notion. This hypothesis is also confirmed by other mutants from this region (see below). N167Y/N167I mutants leak potassium even though no excessive hydration of TM1/2-TM3 crevices is expected here. All these facts favor the idea that no excess hydration of crevices is needed for potassium permeability even in the wild type MscS, in its inactivated state. On the other hand, the G121A mutant, which exhibits silent inactivation phenotype in vitro and LOF phenotype in vivo, does not leak potassium (Table 1), which favors the need of the hydration of crevices.

However, it is possible that both mutations result in channels that preferentially inactivate to different conformations. Indeed, it was shown that MscS dwells at least into two adapted states, desensitized and inactivated (11). Consistently, there is also an apparent difference in the activities of both mutants; N167Y almost did not open at all (supplemental Fig. S3), whereas the G121A mutant opened normally but went into an inactivated state “silently” from the closed state (11).

In addition to GOF and LOF channels mentioned above, we discovered a few mutants that have normal threshold of activation but do not adapt to pressures. These mutant channels are N117K and G168D, and they have mutations in the TM3-\(\beta\) domain interface resulting in increased polarity of the interface. Thus, their properties are opposite to the N167I/N167Y LOF mutant. Adaptation is a complex process involving reversible desensitization followed by complete inactivation, which are reflected structurally in the kinks at Gly-121 and Gly-113, respectively (11). Pressure protocols used in this work did not allow us to distinguish which of these two processes is affected in N117K and G168D mutants. From simple geometric considerations, it seems plausible that the inactivated state of the channel, characterized by the TM3 kink at Gly-113, could be affected more by these mutations because there is more exposure of TM3b to the \(\beta\) domain in this state. Moreover, Asn-117 (with Gly-168, with which it interacts and whose mutation has the opposite effect of the mutation at Asn-117) is located between Gly-113 and Gly-121, in the region that is proposed to change its position during transition from the desensitized to the inactivated state. The kink at Gly-121 is a feature not only of the proposed desensitized state but also of the closed state. We found that the threshold

FIGURE 6. Covalent cross-linking of TM3b and the \(\beta\) domain locks the channels in a nonconducting state. A, single N117C and G168C mutants are active under ambient conditions. B, activity of the double N117C/G168C mutant is not visible (control) unless DTT (2.5 mM) is added (DTT). Washing out DTT results in loss of MscS activity, and only MscL channels are active (wash-out). The illustration below shows that the formation of an S–S bridge is possible in nonreducing conditions in the N117C/G168C mutant only. The double N117C/G168C mutant was expressed in the MJF429 strain that retains MscL channel activity. Channel openings are visible as upward deflections from the closed (c) level. Corresponding traces of pressure and current are shown in the same color (black, blue, or red).
of G168D activation is similar to that of the wild type MscS (data not shown), suggesting that binding of β domain to TM3 does not contribute energetically to the stability of closed state. On the other hand, using double cysteine mutant N117C/G168C, which does not open unless a reducing agent is present, we provided evidence that TM3 and β do change their inter-domain positions during the closed to open transition. Altogether, we propose that the N117K and G168D mutations weaken the interaction between TM3 and the β domain, supporting the model in which TM3b is bent away in the open state and bound to various degrees to the β domain in the closed, desensitized, and inactivated states.

Inspection of an alignment of MscS homologs revealed that amino acids forming the TM3-β domain interface are functionally conserved, and their substitutions are correlated (supplemental Fig. S5). For instance, EcMscS and several of its homologs have Gln-112 and Gly-113 on TM3. However, in other homologs, Gly-113 is substituted by negatively charged aspartate or glutamate. In the latter cases, correlated substitutions to positively charged lysine or arginine can be found either at position 112 on TM3 or at 168 at the β domain resulting in proteins with amino acid pairs Asp(Glu)-113/Lys(Arg)-112 and Asp(Glu)-113/Lys(Arg)-168 (supplemental Fig. S5A). According to previous reports, the G113E mutation results in a noninactivating MscS channel (11). This result is confirmed by the presence of glutamate at position 113 in the noninactivating MscK, another bacterial mechanosensitive channel, and all its homologs (supplemental Fig. S5B). According to our model, binding of TM3b to the β domain is crucial for inactivation. Because MscK and its homologs do not inactivate, why in their sequences is the positively charged Lys-168 (or Arg-168) correlated to Glu-113? The correlated changes in TM3 and the β domain of positively and negatively charged amino acids in MscS and MscK homologs resemble the pair N117K (TM3b) and G168D (β domain) studied in our work. Mutants N117K and G168D are potassium-leaking mutants, but the double mutant N117K/G168D is not leaky to potassium. We think that, similarly to the double N117K/G168D mutant, the tight seal of noninactivating MscK homologs could be ensured by the Glu(Asp)-113/Lys(Arg)-168 pair. We propose that binding of the β domain to TM3b is important not only during channel inactivation but also ensures the tight closure of all channels from the MscS family. The evolutionary pressure against leaky channels resulted in functional conservation of the interaction of TM3 and the β domain, and this is why these fragments of protein are the most conserved parts in all members of the MscS family.

In summary, from the analysis of the distribution of the mutations and from the analysis of the mutant properties, we infer that the common feature of potassium-leaking mutants are weakened interactions of TM3 and surrounding structures. Our screen revealed that the tight seal of TM3 pore helices depends not only on their own but is also determined by the TM1/TM2 paddle (attached to TM3 in the closed and open states) and the β domain (attached in closed and desensitized/inactivated states) (Fig. 3C). It is their positions that force the TM3 gate to keep shut. Our model of TM3-β domain interaction parallels previous papers that suggested involvement of the cytoplasmic domain in gating (17, 19, 21, 22). Binding/unbinding of β domain to TM3b could influence activity of the channel but could also be the mechanism by which structural changes in the transmembrane domain are transmitted to changes in the cytoplasmic domain.

Acknowledgments—We thank Professor Brad Rothberg (Temple University School of Medicine, Philadelphia) and Professor Evert Bakker (University of Osnabruck, Germany) for kindly providing E. coli strains TK2446 and LB2003 and Professor Ian R. Booth (University of Aberdeen, United Kingdom) for E. coli strains MJF429 and MJF465.

REFERENCES

1. Levina, N., Tötemeyer, S., Stokes, N. R., Louis, P., Jones, M. A., and Booth, I. R. (1999) EMBO J. 18, 1730–1737
2. Bass, R. B., Strop, P., Barclay, M., and Rees, D. C. (2002) Science 298, 1582–1587
3. Wang, W., Black, S. S., Edwards, M. D., Miller, S., Morrison, E. L., Bartlett, W., Dong, C., Naismith, J. H., and Booth, I. R. (2008) Science 321, 1179–1183
4. Vásquez, V., Sotomayor, M., Cortes, D. M., Roux, B., Schulten, K., and Perozo, E. (2008) J. Mol. Biol. 378, 55–70
5. Vásquez, V., Sotomayor, M., Cordero-Morales, J., Schulten, K., and Perozo, E. (2008) Science 321, 1210–1214
6. Sotomayor, M., and Schulten, K. (2004) Biophys. J. 87, 3050–3065
7. Anishkin, A., and Sukharev, S. (2004) Biophys. J. 86, 2883–2895
8. Sotomayor, M., van der Straaten, T. A., Ravaioili, U., and Schulten, K. (2006) Biophys. J. 90, 3496–3510
9. Sotomayor, M., Vásquez, V., Perozo, E., and Schulten, K. (2007) Biophys. J. 92, 886–902
10. Spronk, S. A., Elmore, D. E., and Dougherty, D. A. (2006) Biophys. J. 90, 3555–3569
11. Akitake, B., Anishkin, A., Liu, N., and Sukharev, S. (2007) Nat. Struct. Mol. Biol. 14, 1141–1149
12. Anishkin, A., Akitake, B., and Sukharev, S. (2008) Biophys. J. 94, 1252–1266
13. Anishkin, A., Kamaraou, K., and Sukharev, S. (2008) J. Gen. Physiol. 132, 67–83
14. Belyy, V., Anishkin, A., Kamaraou, K., Liu, N., and Sukharev, S. (2010) Nat. Struct. Mol. Biol. 17, 451–458
15. Edwards, M. D., Li, Y., Kim, S., Miller, S., Bartlett, W., Black, S., Dennison, S., Iscla, I., Blount, P., Bowie, J. U., and Booth, I. R. (2005) Nat. Struct. Mol. Biol. 12, 113–119
16. Miller, S., Edwards, M. D., Ozdemir, C., and Booth, I. R. (2003) J. Biol. Chem. 278, 32246–32250
17. Edwards, M. D., Booth, I. R., and Miller, S. (2004) Curr. Opin. Microbiol. 7, 163–167
18. Akitake, B., Anishkin, A., and Sukharev, S. (2005) J. Gen. Physiol. 125, 143–154
19. Grajkowski, W., Kubalski, A., and Koprowski, P. (2005) Biophys. J. 88, 3050–3059
20. Nomura, T., Sokabe, M., and Yoshimura, K. (2008) Biophys. J. 94, 1638–1645
21. Machiyama, H., Tatsumi, H., and Sokabe, M. (2009) Biophys. J. 97, 1048–1057
22. Koprowski, P., and Kubalski, A. (2003) J. Biol. Chem. 278, 11237–11245
23. Miller, S., Edwards, M. D., Ozdemir, C., and Booth, I. R. (2003) J. Biol. Chem. 278, 32246–32250
24. Ou, X., Blount, P., Hoffman, R. J., and Kung, C. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 11471–11475
25. Okada, K., Moe, P. C., and Blount, P. (2002) J. Biol. Chem. 277, 27682–27688
26. Yoshimura, K., Nomura, T., and Sokabe, M. (2004) Biophys. J. 86, 2113–2120
27. Nomura, T., Sokabe, M., and Yoshimura, K. (2006) Biophys. J. 91,
Genetic Screen for Potassium Leaky MscS Channels

2874–2881
28. Dosch, D. C., Helmer, G. L., Sutton, S. H., Salvacion, F. F., and Epstein, W. (1991) J. Bacteriol. 173, 687–696
29. Parfenova, L. V., Crane, B. M., and Rothberg, B. S. (2006) J. Biol. Chem. 281, 21131–21138
30. Sun, S., Gan, J. H., Paynter, J. J., and Tucker, S. J. (2006) Physiol. Genomics 26, 1–7
31. Irizarry, S. N., Kutluay, E., Drews, G., Hart, S. J., and Heginbotham, L. (2002) Biochemistry 41, 13653–13662
32. Paynter, J. J., Sarkies, P., Andres-Enguix, I., and Tucker, S. J. (2008) Channels 2, 413–418
33. Minor, D. L., Jr., Masseling, S. J., Jan, Y. N., and Jan, L. Y. (1999) Cell 96, 879–891
34. Baurman, E. T., McLaggan, D., Naprstek, J., and Epstein, W. (2004) J. Bacteriol. 186, 4238–4245
35. Li, C., Edwards, M. D., Jeong, H., Jeong, H., Roth, J., and Booth, I. R. (2007) Mol. Microbiol. 64, 560–574
36. Humphrey, W., Dalke, A., and Schulten, K. (1996) J. Mol. Graph. 14, 37–38
37. Miller, S., Bartlett, W., Chandrasekaran, S., Simpson, S., Edwards, M., and Booth, I. R. (2003) EMBO J. 22, 36–46
38. Anishkin, A., and Sukharev, S. (2009) J. Biol. Chem. 284, 19153–19157
39. Yoshimura, K., Batiza, A., Schroeder, M., Blount, P., and Kung, C. (1999) Biophys. J. 77, 1960–1972
40. Akitake, B., Spelbrink, R. E., Anishkin, A., Killian, J. A., de Kruijff, B., and Sukharev, S. (2007) Biophys. J. 92, 2771–2784
41. Tombola, F., Pathak, M. M., Gorostiza, P., and Isacoff, E. Y. (2007) Nature 445, 546–549