Mechanosensitive channels must make a large conformational change during the transition from the closed to the open state. The crystal structure of the open form of the *Escherichia coli* MscS channel was recently solved and depicts a homohexamer (1). In this study, cross-linking of site-specific cysteine substitutions demonstrates that residues up to 10–33 Å apart in the crystal structure readily form disulfide bridges in the closed form and can also be cross-linked by a 10-Å linker. Cross-linking between adjacent subunits stabilizes the heptameric form of the channel providing biochemical evidence to support the crystal structure. The data are consistent with the published model (1) in that the membrane domain is highly flexible and that the closed to open transition may involve a significant displacement of transmembrane helices 1 and 2, possibly by as much as 30 Å. The data are also consistent with significant flexibility of the cytoplasmic domain.

The MscS protein has been much less intensively studied as a result of the relatively recent discovery of the structural gene (12). Genetic and biochemical analyses established that the protein has three transmembrane (TM) helices and a large carboxyl-terminal cytoplasmic domain (19). This was confirmed by resolution to 3.8 Å of a crystal structure of the MscS channel that also showed the protein to be a homohexamer (1). The large cytoplasmic domain was demonstrated to form a 40-Å diameter chamber perforated by eight holes, seven of which arise at the boundary between pairs of monomers and the eighth from the formation of a β barrel by the carboxyl-terminal 15 residues. In the crystal structure the TM3 helix bends at Gly113 such that the carboxyl-terminal part of this helix lies parallel to the membrane surface. The loop between TM2 and TM3 (residues 91–95) forms an extended chain that is part of the wall of the open pore. Helices TM1 and TM2 are close packed to each other but are displaced away from TM3. It has been suggested that channel closure might be effected by closer packing of TM1 and TM2 with TM3 with propagation of this change through the TM2-TM3 loop into TM3 and the cytoplasmic domain (1). Here we present evidence that in the closed channel the transmembrane helices and residue 267 at the base of the carboxyl-terminal domain can be in close proximity to each other. These data support recent electrophysiological data suggesting that a rearrangement of the carboxyl-terminal domain is essential for channel gating (14).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**

*E. coli* strains MJF455 (MscL*MscS*) and MJF465 (MscL*MscSMcK*) were used throughout for this analysis and have been described previously (13). Plasmids pMscS and pMscSH6 were described previously (13).

**Growth and Downshock Protocols**

Strains were grown routinely at 37 °C in Luria-Bertani medium (LB) containing per liter: 10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract. Medium was supplemented with ampicillin (25 μg/ml) if required. Agar plates contained 1.4 g/liter agar. Analysis of survival after downshock was essentially as described previously (13). Survival assays were performed in MJF465, and both induced and uninduced cell preparations were tested.

**Mutant Creation**

The single Cys mutants were created using the Stratagene QuickChange site-directed mutagenesis protocol using the primers listed in Table I. Six single Ser to Cys mutants at residues 9, 26, 49, 58, 95, and 267 were created in pMscSH6. The mutant plasmids were verified by sequencing on both strands and were transformed into MJF465. Mutant S267C was also constructed in pMscS to eliminate potential interference in the analysis by the His tag.

**Membrane Preparation and Western Blotting**

Membranes harvested from transformants that had been induced for 30 min using 0.3 mM IPTG as described previously (13) were resus-
pended in phosphate-buffered saline containing 137 mM NaCl, 2.7 mM RCl, 10 mM NaH2PO4, 1.4 mM K2HPO4, pH 7.4 and Complete™ EDTA-free protease inhibitors (Roche Diagnostics) (15). Protein concentration in membrane preparations was assayed by the Folch-Ciofide method (16). Western blots were performed as described previously using either anti-His6 antibodies (mouse IgG2a isotype) (Sigma) or peptide antibodies-specific for MscS (13). Preformed SDS-polyacrylamide gels (Novex) were used to separate the proteins prior to transfer onto nitrocellulose.

Purification of MscS

Membrane preparations derived from cells expressing the S267C mutant (1.5 mg/ml membrane protein) were incubated with 31.25 μM oPDM for 15 min at 25 °C and the reaction stopped by the addition of DTT (5 mM final concentration). A sample was retained for Western blotting to verify that reaction with oPDM was as observed in earlier experiments. The treated membranes were harvested by centrifugation (50,000 rpm, Beckman TLA100.4 rotor, for 1 h at 4 °C) and the pellets weighed to calculate the total protein present. The pellets (~0.2 g) were suspended in 5 ml of 50 mM sodium phosphate buffer, pH 7.4, containing 300 mM NaCl, 50 mM imidazole, and 2% Triton X-100 (membrane grade, Roche Diagnostics). The membranes were incubated at 37 °C for 2 h and were shaken at intervals. The solubilized protein was centrifuged (50,000 rpm, Beckman TLA100.4 rotor, for 1 h at 4 °C) and the clear supernatant retained. In previous experiments it had been established that 2% Triton X-100 gave efficient solubilization (~95%) of MscS (data not shown). The supernatant was mixed with 0.25 ml nickel-nitritolriacetic acid-agarose (Qiagen), placed in Qiagen 1 ml polypropylene columns, and the columns were capped and placed on a rocking platform at room temperature for ~16 h. The columns were then washed four times with 2.5 ml of 50 mM sodium phosphate buffer, pH 7.4, containing 300 mM NaCl, 50 mM imidazole, 10% glycerol, 10% ethanol, 10 mM β-mercaptoethanol, and 0.2% membrane-grade Triton X-100. The buffer also contained 1 Complete™ EDTA-free protease inhibitor tablet (Roche Diagnostics) per 50 ml of solution. The membrane protein was eluted by addition of four 0.25 ml volumes of the above wash buffer amended to contain 0.3 mM imidazole. Eluted protein fractions were treated to remove excess detergent prior to gel electrophoresis using the Pierce PAGEprep™ product following the manufacturer’s instructions. Samples were separated on Novex NuPAGE 4–12% BisTris gels run in MES buffer.

Protein bands corresponding to the monomer through to the hexamer were excised from the SDS-PAGE gels and were reduced, S-alkylated, and in-gel-digested with trypsin (Promega, Madison, WI). The tryptic peptides were extracted from the gel piece, and an aliquot of the mixture was desalted using a GELEOader tip containing POROS R2 sorbent (PerSeptive Biosystems). The peptides were then eluted onto a sample plate with 0.5 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma, Poole, UK) in 70% acetonitrile, 5% formic acid. Mass spectra were acquired with a PerSeptive Biosystems Voyager DE-STR MALDI-TOF mass spectrometer operated in reflectron-delayed extraction mode. Spectra were calibrated with trypsin auto-digestion products and proteins identified by interrogation of protein databases using MASCOT and MS-Fit data base searching programs.

Cross-linking Reactions

All cross-linking protocols were based on published methods (17, 18). Copper/Phenanthroline—Prewarmed (37 °C) membranes, containing 1.5 mg of membrane protein/ml, were treated with freshly prepared copper/phenanthroline reagent at 37 °C for 10 min. The final concentration of Cu2+ and o-phenanthroline were 0.167 and 0.5 mM, respectively. The reagent was made by mixing equal volumes of freshly prepared 30 mM phenanthroline (dissolved in ethanol) and aqueous 10 mM CuSO4. This stock was diluted to give the appropriate concentrations of Cu2+ and o-phenanthroline (see above). Control incubations were performed with ethanol. Reactions were stopped by addition of NEM (10 mM final concentration).

MTS—MTS—This reaction was dissolved in Me2SO to provide a 1 mM stock solution and was used at a final concentration of 100 μM. Reactions were incubated at 25 °C for 1 h and quenched with NEM (as above). Controls contained an equivalent volume of Me2SO. o-Phenylendiaminediaimde—o-PDM was dissolved in Me2SO to provide a 250 mM stock solution, which was kept at room temperature in a foil-wrapped tube and was discarded after each experiment. The final concentration of o-PDM was between 15 and 250 μM. Similar data were obtained at all concentrations of o-PDM and 31.25 μM o-PDM was adopted for routine analysis of cross-linking. Membranes (1.5 mg of protein/ml) were incubated at either 4 or 25 °C for up to 1 h. The reaction was terminated by addition of DTT (5 mM final concentration).

For cross-linking in whole cells, strains MJF465/mescSH wild type and Cys mutant S267C were grown to mid-exponential phase (OD650 = 0.5) in minimal medium (13) containing 0.3 mM NaCl with IPTG (0.3 mM) present for 30 min prior to addition of o-PDM (100 μM final concentration). Cells were incubated for 20 min in o-PDM and the reaction quenched with 5 mM DTT. For direct detection of MscS in whole cells, 15–25 μg of total cell protein (equivalent to 100–170 μl of cell suspension at OD650 = 0.6) in Laemml loading buffer (19) containing 2% β-mercaptoethanol were loaded onto a Novex NuPAGE 4–12% BisTris gel and run in MES buffer. Western blotting was carried out as described previously using anti-His6 antibodies. To prepare membranes from the o-PDM-treated cells, approximately 17 ml of cell suspension (OD650 = 0.6) was harvested by centrifugation (4000 rpm, duan MR222, 10 min, 4 °C) and suspended in cold phosphate-buffered saline containing Complete™ EDTA-free protease inhibitors (Roche Diagnostics). Cells were lysed by French press (18,000 lb/inch2) and the membranes harvested by centrifugation as described previously (13).

Results

Creation and Physiology of Single Cysteine Mutants of MscS—Single cysteine mutants were created in the 286-residue E. coli MscS protein, which is Cys-free, by replacing selected serine residues (Ser5, Ser28, Ser34, Ser28, Ser28, and Ser207) with cysteine. Residues Ser5 and Ser28 are not visible in the crystal structure, which starts at Tyr27. However, we can estimate the separation of S28C residues from the position of Tyr27. In adjacent subunits of the crystal structure, which is proposed to depict the open channel, the mutated residues are 10–33 Å apart (Fig. 1a). Both basal and induced expression of the mutant proteins protected the triple MS channel-deficient mutant (MJF465; mscL mscK mscS−) (12) against downshock (Fig. 1b; data not shown for induced channels as this was found to be 100% in all cases). Protection by the basal expression of the MscS channel is a strong indicator of normal organization and function in the channel (13). The resultant proteins were expressed at similar levels to the Cys-free parent after induction with 0.3 mM IPTG (Fig. 2, a and b). These data suggest that the cysteine mutations do not significantly disturb the MscS channel organization. In non-reducing gels S9C mutants were detected as dimers, even prior to incubation with an oxidising agent (Fig. 2a), but were converted to monomers by DTT (Fig. 2b). Neither S26C nor S95C, which are also located on the periplasmic face of the channel, were observed as dimers in the absence of oxidising agents. In the crystal structure, S9C is not visible (1) but is predicted to be periplasmic (13, 20). It alone has sufficient conformational plasticity to form disulfide bridges without addition of any oxidizing agent.

Cross-linking by Oxidizing Agents—Treatment of membrane preparations, in which the channel is in the closed state (21), with copper/phenanthroline reagent (18) generated dimers for all of the proteins except the wild type, which is a Cys-free protein, and S49C (Fig. 2a). The monomer and dimer of MscS have slightly faster mobility than expected from the theoretical
MscS proteins detected by Western blotting using anti-His6 tag primary run in MES buffer), transferred onto nitrocellulose membranes, and observed more often than the dimer and the dimer was only visible on longer exposures of the Western blot. All of the disulfide bridges could be reduced by incubation with DTT except that for S95C, which proved to be quite resistant to the reducing agent (Fig. 2b). Incubation with the disulfide bridge-generating reagent MTS-1-MTS (17) also resulted in dimer formation for all the Cys mutants, except S49C, and incubation with DTT reduced the dimers to the monomer (data not shown).

Cross-linking with oPDM—oPDM cross-links cysteine residues that are capable of adopting positions within ~10 Å of each other (range 9.4 ± 0.4 Å) (22). Exposure of the MscS single Cys mutants led to S26C, S58C, S95C, and S267C forming higher order oligomers that were stable to SDS (Fig. 3a). Only S95C formed solely the dimer, whereas tetramers and hexamers were evident for S26C on extended exposure of the Western blot and the dominant form obtained with S58C had the mobility of the tetramer (Fig. 3a). The most startling result was seen with S267C, which formed multiple oligomeric forms from dimer through to the heptamer (Fig. 3a). The multimeric forms were seen to proceed via formation of the dimer followed by the appearance of trimers, then tetramers, pentamers, and finally the heptamer and hexamer (Fig. 3b). This observation was only made with the S267C mutant after incubation with oPDM. All of the cross-linked forms observed with S267C after oPDM treatment exhibited unusually fast mobilities suggesting that the protein might exhibit a more compact conformation. Incubation at high temperature or with urea-containing loading buffer, immediately prior to loading the gel, did not breakdown the multimers. These data give the clearest support to the heptameric form seen in the crystal structure (1) and suggests that this organization is maintained in the closed state.

The observation of the heptamer was made prior to the crystal structure being available, at a time when the protein was thought to be hexameric (21). This led us to perform a number of controls to eliminate the possibility of artifacts. The cloned MscS protein has a carboxyl-terminal His-tag to facilitate detection and purification. To ensure that this organization is maintained in the closed state.

The observation of the heptamer was made prior to the crystal structure being available, at a time when the protein was thought to be hexameric (21). This led us to perform a number of controls to eliminate the possibility of artifacts. The cloned MscS protein has a carboxyl-terminal His-tag to facilitate detection and purification. To ensure that this organization is maintained in the closed state.
tween subunits of the closed channel, reactions were performed in growing cells where the channel must be closed. Bands up to the heptamer were visible in Western blots of whole cells and in membranes purified from the cross-linked cells (Fig. 4b).

The wild type protein, which lacks cysteine residues, was not affected by oPDM, which suggests that the observed pattern of oligomers was not caused by reaction of the cross-linker with other residues. *E. coli* cells express a MscS-related protein MscK that contains a number of cysteine residues. However, the results shown here were obtained in MJF465, which lacks MscK, and identical patterns of cross-linking were observed in strains that possessed MscK. To determine whether the higher molecular mass oligomers arose by cross-linking to other proteins, we performed tryptic peptide fingerprinting on the purified protein. The MscS S267C protein was purified by Ni²⁺-chelate chromatography after treatment of the membranes with oPDM and the purified material resolved by SDS-PAGE. A clear pattern of seven bands was seen (Fig. 4c). Peptide fingerprinting by MALDI-TOF mass spectrometry demonstrated that no other protein was linked to MscS in any of the seven bands. Mowse scores of >1e + 005 were observed for MscS in all seven protein bands, with no other significant hits. All seven proteins gave similar tryptic digest patterns, suggesting that no further cross-links were formed in the higher order oligomers. Furthermore, no evidence was found for other intra-MscS cross-links that could account for the higher order oligomers. Eleven of the 16 potential tryptic peptides were observed. Two of the missing peptides correspond to large (4.2 and 4.9 kDa) molecular mass hydrophobic peptides (TM1 and TM3) that are not readily detected by mass spectrometry. One of the missing peptides contained the cross-linked residue, S267C, and could be detected as an oPDM-modified peptide, but the cross-linked peptide itself was not observed in the MS. These data support a model in which the oligomeric state is probably formed by stabilization of the multimeric state rather than by additional cross-linking. This suggestion is supported by the observation that oxidation of single cysteine residues in several mutants also stabilized higher order oligomers (Figs. 2a and 3a). The native MscS protein, when expressed to very high levels in *E. coli*, also forms multiple molecular forms up to the heptamer, suggesting that this property is intrinsic to MscS and that cross-linking merely stabilizes this tendency.

**DISCUSSION**

The analysis reported here is of cross-linking of specific residues in the closed state of the MscS channel. Previous workers have established that MS channels in isolated membranes are in the closed state (11). Most of our studies were conducted on isolated membranes and represent analysis of the closed state. Additional studies showed that identical patterns of cross-linking of S267C were observed in isolated membranes and in whole cells, where for physiological reasons the channel must be closed (Fig. 4). MscL represents the most in-depth analysis of the closed and open configurations of an MS channel, with the former observed in the crystal state and the latter by molecular dynamics simulations (6, 8, 10, 23). All the models envisage the transition from a compact protein to an expanded state as the channel moves from the closed to the open state. Cross-linking data has been used extensively to support these models. In the MscS crystal structure, which is proposed to represent the open state of the channel (1), the distance between the residues selected for mutagenesis range from 10 to 33 Å (Fig. 1a). None should easily form disulfide bonds, yet the majority do so in the closed state of the channel (Fig. 2a). Our data suggest that spontaneous cross-linking, which could trap a rare conformation of a flexible MscS protein, does not take place to any significant extent except for S9C (Fig. 2).

---

*R. Bass, personal communication.*
latter may be a substrate for the periplasmic disulfide oxidoreductase system (24). Conformational flexibility is known to influence the apparent distance between residues, as revealed by cross-linking experiments (25). Generally it is considered that proximity and cross-linking efficiency are correlated, but if a polypeptide chain exhibits significant flexibility then residues can be cross-linked, although far apart in the native structure.

Most of the mutants were cross-linked by oPDM, suggesting that these residues can readily adopt positions within the distance required for efficient cross-linking. oPDM has been estimated to bridge 9.4 ± 0.4 Å (22) and, with the possible exception of S95C, would be expected to react poorly with the Cys mutants in the open channel configuration. The only residue that did not form either disulfide bridges or react with oPDM, S49C, is predicted to be the least favorably arranged for cross-linking. The crystal structure suggests that the S49C side chains are not positioned in a way that would easily facilitate reaction. The lack of reactivity of S49C provides an internal control for nonspecific cross-linking, since the absence of cross-links involving this residue under any of the conditions tested suggests that those that are formed by other residues are specific and a realistic indication of proximity or dynamics in the closed structure. S58C readily formed disulfides with copper/phenanthroline and cross-linked oligomers with oPDM, suggesting that in the closed structure these residues can be located as few as ~3 Å apart compared with up to ~33 Å in the crystal structure.

Rees and colleagues (1) proposed a model for the gating of MscS that envisaged a significant lateral movement within the plane of the membrane during the transition from the closed to the open state. The data presented here are consistent with such a model, since residues that are distant in the crystal are cross-linked in a manner predicted from the model. In this case, the cross-linking data would predict that transmembrane helices 1 and 2 undergo a displacement of up to 30 Å during the transition from the closed to the open state. The most COOH-terminal mutant studied, S267C, lies at the base of the large carboxyl-terminal domain of the channel, at the boundary between the αβ domain and the final β barrel that completes the protein. Adjacent S267C residues are separated by ~20 Å in the crystal structure but readily form disulfide bridges and are cross-linked by oPDM (Fig. 3, a–c). These data suggest that the cytoplasmic domain may undergo a significant conformational change during the closed to open transition. Support for this suggestion comes from the observed inhibition of gating of MscS by cross-linking of carboxyl-terminal His tags by Ni²⁺ (14). The inhibition required high concentrations of Ni²⁺, suggesting that multiple cross-links were needed to block the re-organization of the carboxyl-terminal domain. Second, small deletions affecting the carboxyl terminus of MscS prevent the stable insertion of the protein into the membrane, consistent with the importance of this domain for the structure of the channel (13).

The data presented here strongly support the published model for MscS, which is derived from the crystal structure (1) and supporting genetic work (13). The cross-linking of S267C provides the first biochemical evidence for the heptameric structure of MscS. The progressive formation of higher oligomers, most evident with S267C treated with oPDM, suggests that the MscS protein can form SDS-stable complexes similar to those seen for K⁺ channels (26, 27). The cross-linking data, particularly for S267C, suggest that when two subunits are linked by an inflexible bond, the interactions with other subunits are strengthened, i.e., a conformational change consequent upon cross-linking of pairs of subunits is transmitted to adjacent subunits such that higher oligomers become stable. The rate of dimer formation is rapid relative to the formation of non-covalently linked oligomers (0.5–3 min compared with 9.5–12 min, although the trimer is evident after 3-min incubation), suggesting that a significant conformational change is required for the stabilization of the contact between the subunits. In conclusion, we believe our data support the heptameric model for MscS and suggests significant flexibility in the protein that may be essential for the transition between the closed and open states.

Acknowledgments—We greatly appreciate discussions with W. Bartlett, R. Bass, D. C. Rees, P. Blount, C. J. Deutsch, N. A. Booth, and L. Stanfield. We also thank The Aberdeen Proteomics Unit (I. Davidson and E. Stewart) for their proteomics work and for helpful discussions.

REFERENCES
1. Bass, R. B., Strop, P., Barclay, M., and Rees, D. C. (2002) Science 298, 1582–1587
2. Sackin, H. (1995) Annu. Rev. Physiol. 57, 333–353
3. Booth, I. R., and Louis, P. (1999) Curr. Opin. Microbiol. 2, 166–169
4. Blount, P., Sukharev, S. I., Moe, P. C., Martinac, B., and Kung, C. (1999) Methods Enzymol. 294, 458–482
5. Sukharev, S. I., Martinac, B., Arshavsky, V. Y., and Kung, C. (1999) Biophys. J. 65, 177–183
6. Sukharev, S., Betanzos, M., Chiang, C. S., and Guy, H. R. (2001) Nature 409, 720–724
7. Perezo, E., Cortes, D. M., Somporphinisut, P., Kldoa, A., and Martinac, B. (2002) Nature 418, 942–948
8. Chang, G., Spencer, R. H., Lee, A. T., Barclay, M. T., and Rees, D. C. (1998) Science 282, 2220–2226
9. Perezo, E., Kldoa, A., Cortes, D. M., and Martinac, B. (2002) Nat. Struct. Biol. 9, 696–703
10. Kung, Y. F., Shen, Y. F., Warth, T. E., and Ma, J. P. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5999–6004
11. Betanzos, M., Chiang, C. S., Guy, H. R., and Sukharev, S. (2002) Nat. Struct. Biol. 9, 704–710
12. Levine, N., Totemeyer, S., Stokes, N. R., Louis, P., Jones, M. A., and Booth, I. R. (1999) EMBO J. 18, 1730–1737
13. Miller, S., Bartlett, W., Chandrasekaran, S., Simpson, S., Edwards, M., and Booth, I. R. (2003) EMBO J. 22, 36–46
14. Koprowski, P., and Kabalski, A. (2003) J. Biol. Chem. 278, 11237–11245
15. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., p. B.12, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
17. Wu, J. H., and Kaback, H. R. (1977) J. Mol. Biol. 107, 285–293
18. Bass, R. B., and Falke, J. J. (1999) Struct. Fold. Des. 7, 829–840
19. Laemmli, U. (1970) Nature 227, 680–685
20. McGlashan, D., Jones, M. A., Gousebett, G., Levine, N., Lindsey, S., Epstein, W., and Booth, I. R. (2002) Mol. Microbiol. 43, 521–536
21. Sukharev, S. (2002) Biophys. J. 83, 290–298
22. Green, N. S., Reiser, E., and Houk, K. N. (2001) Protein Sci. 10, 1293–1304
23. Sukharev, S., Durell, S. R., and Guy, H. R. (2003) Biophys. J. 81, 917–936
24. Hiniker, A., and Bardwell, J. C. A. (2003) Biochemistry 42, 1179–1183
25. Falke, J. J., and Koshland, D. E. (1987) Science 237, 1596–1600
26. Cortes, D. M., and Perezo, E. (1997) Biochemistry 36, 10343–10352
27. Heginbotham, L., Odessey, E., and Miller, C. (1997) Biochemistry 36, 10335–10342
28. Martz, E. (2002) Trends Biochem. Sci. 27, 107–109
The Closed Structure of the MscS Mechanosensitive Channel: CROSS-LINKING OF SINGLE CYSTEINE MUTANTS

Samantha Miller, Michelle D. Edwards, Cafer Ozdemir and Ian R. Booth

J. Biol. Chem. 2003, 278:32246-32250.
doi: 10.1074/jbc.M303188200 originally published online May 26, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303188200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 27 references, 8 of which can be accessed free at http://www.jbc.org/content/278/34/32246.full.html#ref-list-1