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Stoichiometry of the Large Conductance Bacterial Mechanosensitive Channel of *E. coli.* A Biochemical Study

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Abstract. MscL, a 15 kDa transmembrane protein, is the only component involved in the formation of a 3 nS channel in the inner membrane of *Escherichia coli* that opens in response to mechanical or osmotic stress. While previous data had suggested that the functional MscL complex might be a hexamer, a recent crystallographic study of the MscL homologue from *M. tuberculosis* reveals a pentameric structure. The present work further examines the stoichiometry of the *E. coli* MscL using a variety of biochemical approaches. Detergent-purified 6His-MscL in solution and MscL in the membrane could be chemically crosslinked with the products displaying ladderlike patterns on SDS gels. Three crosslinking agents (EDC, DMS, and DMA) used at saturating concentrations invariably generated pentamers as the largest product. DSS produced additional bands corresponding to larger complexes although the pentamer band appeared to be the predominant product at high levels of crosslinker. It is not clear whether these extra bands reflect a difference in the crosslinking chemistry of DSS or whether its spacer arm is the longest of those used, or a combination of both facts. For the detergent-solubilized 6His-MscL both sedimentation equilibrium and gel chromatography showed the presence of multiple species. Thus the longer spacer arm could permit both intra- and intercomplex linkages. Nonetheless, the patterns obtained with all agents are consistent with and strongly suggest a pentameric organization for the MscL channel. Expression of MscL as genetically engineered double or triple subunit tandems yields low numbers of functional channels as compared to expressed monomers. The double-tandem assemblies must have an even number of subunits and crosslinking in the membrane confirmed hexamerization. Gel chromatography clearly demonstrated that the channels formed from the double tandems were larger than those formed from WT MscL, consistent with the native channel being pentameric. The observation that both double and triple tandems form channels of normal conductance implies that the pentameric assembly is to some degree independent of the number of subunit repeats in the polypeptide precursor. The channel is thus a pentameric core with the 'extra' subunits left out of the functional complex. From sedimentation equilibrium and size-exclusion chromatography, we also conclude that MscL complexes are not in a dynamic equilibrium with monomers, but are preassembled; and thus, their gating properties must result from changes in the conformation of the entire complex induced by the mechanical stress.

Key words: *Escherichia coli* — Mechanosensitive channel — Subunit assembly — Covalent crosslinking — Multimeric structure

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Abbreviations: MS, mechanosensitive; MscL, mechanosensitive channel of large conductance; OG, β-Octyl Glucopyranoside (Octylglucoside); IPTG, Isopropyl-β-D-Thiogalactopyranoside; ORF, open reading frame; DSS, disuccinimidylsuberate; EDC, 1-Ethyl-3-(Dimethylaminopropyl) carbodiimide; DMS, Dimethylsuberimidate; DMA, Dimethyl adipimidate; FPLC, fast protein liquid chromatography.

Introduction

Mechanosensation is ubiquitous in living organisms and encompasses a wide range of phenomena from microbial osmoadaptation to gravitropism in plants and hearing

and balance in animals. In many systems, electrophysiological data or ion flux measurements indicate that mechanical stress applied to whole cells or membrane patches results in an immediate increase of ionic conductance or permeability which implies direct activation of mechanosensitive (MS) ion channels. Activities of MS channels have been well documented in both specialized sensory cells (Howard et al., 1988) and in many types of nonsensory cells (Sachs & Morris, 1998; Sakin, 1995). A genetic dissection of mechanosensory responses in the nematode *Caenorhabditis elegans*, clearly indicated channel-like participants, along with cytoskeletal and extracellular matrix components, as necessary for a behavioral reflex (Tavernarakis & Driscoll, 1997), but the electrophysiological characterization of this cloned channel complex has not yet been performed. A broad range of phenomenology related to mechanosensation has been observed by patch-clamp techniques combined with some pharmacological data (Hamill & McBride, 1996), yet the molecular constituents of only two MS channels have been identified to date. These are the bacterial mechanosensitive channels of large conductance, MscL (Sukharev et al., 1994) and the recently reported mammalian S-like channel TREK (Patel et al., 1998). The identification of the molecular constituents of these channels now allows the combined application of molecular, biochemical and biophysical techniques for their studies.

The development of techniques that allow for patch-clamping of prokaryotes revealed MS channels in the cell envelopes of gram-negative (Martinac et al., 1987) and gram-positive bacteria (Zoratti & Szabo, 1991) and even in archaea (Le Dain et al., 1998). Three types of MS channel activities have been recorded on giant spheroplasts of *Escherichia coli*, one of larger, 3 nS conductance (called MscL for Mechanosensitive Channel of Large conductance), one of smaller, c.a. 0.8 nS conductance called MscS (Martinac et al., 1987, Sukharev et al., 1993), and one of yet smaller conductance, c.a. 0.3 nS called MscM (Berrier et al., 1996). The MS channels in giant spheroplasts have been shown to gate in response to osmotic gradients (Martinac et al., 1992; Cui, Smith & Adler, 1995) and are implicated in mediating effluxes of osmolytes (Berrier et al. 1992, Blount, Schroeder & Kung, 1997) and even small proteins (Ajouz et al., 1998) from bacterial cells as adaptive reactions to abrupt osmotic shocks.

Using the ability to isolate MscL with detergents and to demonstrate function upon reconstitution into liposomes, we have correlated the MscL activity with the presence of a 17 kDa protein band, and have cloned the corresponding gene, *mscL* (Sukharev et al., 1994*a*). The *mscL* ORF predicts a unique 136 amino acid (15 kDa) protein with two hydrophobic domains and a hydrophilic C-terminus. Expression of the gene was shown to be both necessary and sufficient for MscL activity (Sukharev et al., 1994*a*,*b*). Highly purified MscL was functional when incorporated into artificial liposomes indicating that only one type of polypeptide chain is involved in channel formation, and that the force which gates the channel is transmitted directly via the lipid bilayer (Häse, LeDain & Martinac, 1995; Blount et al., 1996). The recent report of an X-ray structure (to 3.5 Å resolution) for the MscL homologue from *Mycobacterium tuberculosis* (Chang et al., 1998) makes MscL an excellent model system for studies of the molecular mechanism underpinning mechanosensation.

In the present work, the stoichiometry and stability of *E. coli* MscL is examined using biochemical approaches. The uniquely large conductance of MscL (~3 nS) and apparently large diameter of its pore (3.0–4.5 nm, *see* Cruikshank et al., 1997) necessitate that several 15 kDa subunits must be involved in pore formation. The previously proposed hexameric model of MscL assembly (Blount et al., 1996) was based on the use of one type of crosslinking reagent and on the observation that functional channels can be formed as a result of a tandem expression of two MscL subunits linked together. A hexameric assembly was also suggested by the group which attempted a two-dimensional crystallization of MscL (Saint et al., 1998). The most recent X-ray crystallographic studies (Chang et al., 1998) have demonstrated, however, that the MscL homologue from *M. tuberculosis* is a pentamer. Here we report a more extensive crosslinking study which independently suggests that *E. coli* MscL is also a homopentamer. Moreover, by combining expression of MscL as two-subunit tandems with crosslinking and high-resolution size-exclusion chromatography we demonstrate that native MscL is not a hexamer.

We also address the question whether MscL complexes are stable or the assembly has a dynamic character. If the channel has fixed stoichiometry, then gating of the channel would likely entail some conformational changes within the preformed channel. If the stoichiometry is not fixed but dynamic, as proposed by Häse and coworkers (1997), then the mechanism of activation should include stretch-induced recruitment of new subunits into the complex, similar to the mechanism suggested for alamethicin channels (Opsahl & Webb, 1994). The latter case implies that a significant number of monomeric subunits or half-complexes are present in the membrane. Using size-exclusion chromatography and equilibrium sedimentation of mildly solubilized channels we show that MscL monomer is not the component of the membrane and MscL exists as a uniform population of pre-assembled complexes. Parts of this work were published as an abstract (Sukharev, Schroeder & McCaslin, 1999).

Materials and Methods

Strains, Expression Constructs, and Molecular Modifications of the mscL Gene

The *E. coli mscL*-null strain PB104 (Blount et al., 1996), a derivative of AW405, with completely deleted *mscL* ORF and also converted to *recA*-, was used to host all MscL expression constructs. The pB10a-based p5-2-2 plasmid (Sukharev et al., 1994), carrying wild-type *mscL* behind a LacUV promoter, was used as the initial material for all manipulations. Cells were grown in the standard LB medium with ampicillin (100 μg/ml); the *mscL* gene expression was induced by 0.8–1 mM IPTG.

The double MscL tandem (denoted as E2) was generated as previously described (Blount et al., 1996) by engineering an NdeI site at the 5′ end of one clone and the 3′ end of another and linking them together. The triple MscL tandem (E3) was generated by adding NdeI sites on both ends of *mscL* and inserting the third ORF in the middle of the double tandem. The constructs with the correctly oriented insert were determined by restriction analysis.

MscL Purification Using the 6His Tag

To enable one-step biochemical isolation of MscL, a tag of six sequential histidines was added to the C-terminus of MscL by a two-step PCR amplification (Blount et al., 1996). As a result, the predicted molecular mass increased from 14,958 (15 kDa) for wild-type MscL to 15,781 (15.8 kDa) for MscL-6His. The latter was expressed in pB104 cells grown in LB medium in a 40 L fermenter. Cells were French-pressed and the membranes were collected by differential centrifugation. MscL-6His was isolated in nondenaturing conditions from β-octylglucoside-solubilized membranes using a Ni-NTA column (Qiagen, Chatsworth, CA) as described previously (Blount et al., 1996). Briefly, membrane pellets (5–8 g wet weight) were solubilized in 100 ml of extraction buffer containing 300 mM NaCl, 35 mM imidazole (pH 7.2) and 3% OG. The cleared extract was batch-loaded on a Ni-NTA agarose (7 ml bed volume), then the column was packed and extensively washed with the same buffer containing 1% OG. Pure MscL-6His was eluted in several fractions towards the end of a 30 min, 35 to 500 mM linear gradient of imidazole. Five to eight grams of membrane material (wet weight) yielded approximately 0.5–1 mg of MscL-6His protein which was homogeneous by SDS-PAGE.

Size-Exclusion Chromatography

Experiments were performed on a Pharmacia FPLC system equipped with a Superose 6 HR 10/30 column. The column was equilibrated in a 100 mM NaCl, 30 mM Na-phosphate buffer (pH 7.2) and calibrated using protein gel-filtration standards (Bio-Rad, Hercules, CA) at a 0.3 ml/min flow rate. The column was then re-equilibrated with the same buffer supplemented with 1% β-octylglucoside (OG, Calbiochem, La Jolla, CA). Approximately 20 mg (wet weight) of membrane pellet isolated from cells expressing either wild-type MscL (PB 101, ref. 13) or double MscL-MscL tandem was solubilized using a small glass-piston homogenizer in 1 ml of the above buffer supplemented with 3% OG. The extract was cleared by a 10-min centrifugation at 120,000 × *g* on an Airfuge (Beckman), then 0.1 ml was injected onto the column and eluted at 0.3 ml/min flow rate. Small (1.5–0.5 ml) fractions were collected and mixed with a 2× SDS-containing Laemmli buffer. The elution profile of MscL was determined by SDS-PAGE of individual fractions followed by Western blotting (*see below*). A similar size-exclusion experiment was performed with the tag-purified MscL-6His. Fractions containing pure MscL-6His collected from Ni-NTA column were pooled and concentrated 3-fold on a Centricon 30 concentrator (Amicon); 0.2 ml was injected onto the Superose column, and the elution profile was determined by gel electrophoresis of samples from 0.5 ml fractions followed by Coomassie Blue staining.

Analytical Centrifugation

Absorbance contributed by imidazole in the samples was reduced by pooling and concentrating fractions containing purified protein to about 0.2 mg/ml using a Centriplus 30 concentrator (Amicon) and then running this over a PD10 desalting column (Pharmacia) presaturated with 100 mM NaCl, 30 mM Na-phosphate (pH 7.4), 1% (30 mM) OG and 0.3 mM phosphatidylcholine (from egg yolk, Sigma, St. Louis, MO). The presence of phospholipid remarkably stabilized MscL complexes in the detergent solution and enhanced the reproducibility of data. In other experiments, OG was replaced by detergents such as CHAPS (1%), CHAPSO (1%) or dodecylmaltoside (0.2%), (all from Anatrace Maumee, OH) but in the absence of exogenous lipid. In each instance, samples at several initial concentrations were examined by sedimentation equilibrium at several speeds in a Beckman Optima XL-A Analytical Ultracentrifuge (Beckman Instruments, Palo Alto, CA). Because of variability in the amount of residual imidazole and the possible presence of other nonsedimenting, absorbing materials in the sample, water was used as a reference in the centrifuge cell. Data were collected at 280 nm, at 10°C. Sedimentation equilibrium gradients were analyzed by curve fitting, using the nonlinear least squares capabilities of Igor Pro (Wavemetrics, Lake Oswego, OR), to various models including single species, multiple noninteracting and interacting species using approaches similar to those discussed by Laue (1995). For interpretation of the data, we have used a density of 1 g/ml for the solvent; the partial specific volume of MscL was calculated (Cohn & Edsall, 1943) from the composition based on its sequence to be 0.759 ml/gm; the molecular weight of the 6His-tagged monomer was 15,781. Partial specific volumes of octylglucoside and the lipid used were 0.859 and 0.984 ml/g, respectively (Reynolds & McCaslin, 1985), and the molecular weights were 292.4 and 540.

Antibodies and Western Blots

Rabbit antibodies were raised against the C-terminal peptide of MscL: CEIRDLLKEQNNRS (Chiron, San Diego, CA) coupled to a Keyhole Limpet Hemocyanine (Sigma, St. Louis, MO) as described previously (Blount et al., 1996). Antibodies were screened using Western blot procedure against total membrane proteins from wild-type (AW405) strain, overexpresser strain (PB104 carrying p5-2-2 plasmid) as positive controls and PB104 *mscL*$^-$ strain as a negative control. Proteins were separated in 12% SDS-PAGE minigels and electroblotted onto an Immobilon P membrane (Millipore, Bedford, MA). ProtoBlot Western blot AP kit (Promega, Madison, WI) was used according to a complementary protocol.

Covalent Crosslinking In Situ

Approximately 20 mg wet weight of membrane pellets isolated from overexpresser strains (pB104 carrying WT *mscL*, double (pE2) or triple (pE3) tandems) was resuspended in 5 ml of 100 mM NaCl, 30 mM Na phosphate (pH 7.8) and was equally divided among five Eppendorff tubes. A crosslinker, disuccinimidyl suberate (DSS), (Pierce, Rockford, IL), was added to final concentrations indicated in corresponding figure legends. Tubes were gently rotated for 60 min at room tempera-

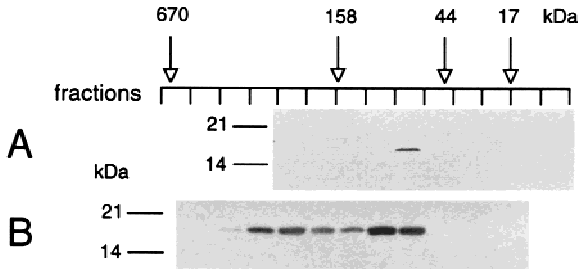

Fig. 1. Size-exclusion chromatography of wild-type MscL under non-denaturing conditions. A total membrane fraction was solubilized with OG and then passed through a Superose 6 HR 10/30 FPLC column with 0.5 ml fraction collected. The position of the MscL peak was determined by Western blot (*A*). A similar experiment was performed with purified MscL-6His. About 20 μg of protein in OG was loaded onto the column and the peak position was visualized on a denaturing Coomassie Blue-stained gel of individual 0.5 ml fractions (*B*). Arrows indicate the elution positions of standard proteins in the absence of OG. The standards used were thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chick ovalbumin (44 kDa) and equine myoglobin (17 kDa). MscL monomers (~15 kDa), if they were present in the mixture would elute in fractions near the 17 kDa mark.

ture, then TRIS buffer (pH 8.0) was added to the final concentration of 100 mM to quench the reaction. Vesicles were washed twice, pelleted a 48,000 × *g* for 20 min and dissolved in 150 μl of Laemmli sample buffer. Proteins were separated in 10–12% minigels and crosslinked products were visualized on Western blots with anti-MscL antibodies.

Crosslinking of MscL-6His In Vitro

Fractions from the Ni-NTA column containing pure MscL-6His were pooled (~15 ml total volume). Since the high concentration of imidazole used in eluting the protein is incompatible with most of the crosslinking agents, the protein was concentrated to about 2.5 ml on a Centriplus 30 concentrator (Amicon) and transferred into the desired buffer by gel-filtration on a PD-10 desalting column (Pharmacia). A buffer consisting of 100 mM NaCl, 30 mM NaPi, pH 7.5 and 1% OG was used with DSS, DMS and DMA. A 3.5 ml of solution containing approximately 1 mg of MscL-6His was diluted additionally with 15 ml of the OG-containing buffer and distributed equally among four or five 5 ml screw-cap tubes. Crosslinker was then added to the desired concentration. After a 60 min incubation at room temperature with gentle agitation, the reactions were quenched by adding 0.5 ml of 1 M Tris pH 8.0. For the EDC crosslinker the buffer recommended by the manufacturer (Pierce) was used and consisted of 100 mM NaCl, 50 mM MES, pH 5.0 and 1% OG. Excess crosslinker was removed by gel-filtration on a PD-10 column pre-equilibrated with the 100 mM NaCl, 30 mM NaPi, pH 7.5 buffer, containing 1% OG. All reaction products were concentrated 20–50-fold on Centricon 30 concentrators, and mixed with appropriate amounts of 2× Laemmli sample buffer. Bands on gels were visualized by silver-stain or Coomassie Blue.

Single-Channel Recording

Giant *E. coli* spheroplasts were generated and used in patch-clamp experiments as described (Martinac et al., 1987). Gene expression in spheroplasts was induced by 1 mM IPTG during the 2-h period of long filament ("snake") formation. The pipette solution contained 200 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM Hepes, pH 7.2; the bath solution was the same plus 0.3 M sucrose. All recordings were performed on excised inside-out patches at −20 mV. Unpolished borosilicate glass pipettes of approximately 2 μm bore diameter were used. The maximal number of channels in patches was assessed by increasing the pressure to 100–200 mm Hg until the open probability reached saturation. Although many patches broke before reaching saturation, the stability was sufficient to observe current plateau in approximately 20% of the attempts. Current traces were recorded using an EPC-7 amplifier (List Electronics) at 10 kHz low-pass filtering and stored on a DAT tape (48 kHz sampling rate). Channel amplitude was assessed off screen using the Fetchex 'Measure' function in the pCLAMP 6 suite (Axon Instruments, Foster, CA).

Results

Characterization of MscL Complexes in Detergent Extracts

As was shown in the previous studies, MscL complexes are relatively stable in mild detergents and are amenable to functional reconstitution in liposomes (Sukharev et al., 1993, 1994) or to covalent crosslinking (Blount et al., 1996). We attempted to determine the molecular size and mass of the native and tag-purified MscL-6His by size-exclusion chromatography and sedimentation equilibrium. The goal was to obtain direct information on the stoichiometry of MscL complexes in detergent solution which might in turn facilitate interpretation of in vitro crosslinking experiments.

To directly assess the size of MscL complexes, size-exclusion experiments were performed using either crude membrane extracts containing unmodified MscL, or purified MscL-6His complexes. Membranes were solubilized in a small volume (0.5 ml) of 3% OG and then the proteins were chromatographed in the presence of 1% OG, which is considered to be a relatively mild detergent. Western blots were used to locate native MscL in small individual fractions that were collected from the FPLC gel-filtration column. Figure 1*A* shows that native MscL emerges as a sharp peak in a single 0.5 ml fraction suggesting that solubilized MscL forms uniformly sized particles. The elution position is at approximately that of a 75 kDa protein based on the column calibration. The particle eluting must have mass contributions from both the MscL subunits and any detergent bound to their hydrophobic surfaces, and thus the mass estimate would support no more than a pentameric assembly of 15 kDa subunits. Size-exclusion chromatography is sensitive to both size and shape of the particles, and thus the estimate of mass may be in error due to differences in the shape between the unknown molecular species and the 'standards' used for calibration, or due to differences in unknown's interaction with the column matrix as compared to the standards. These results clearly demonstrate that the solubilized MscL from membrane preparations exists as a homogeneous popu-

lation, much larger than that expected for the monomer and is likely to be no larger than a pentamer.

Tag-purified MscL-6His was concentrated in lipid-free detergent-containing buffer to about 0.5 mg/ml and examined by size exclusion chromatography. MscL-6His containing particles were found to elute in a broad distribution ranging from 70 to approx. 300 kDa as shown by the Coomassie Blue-stained gel (Fig. 1*B*). Monomeric MscL-6His was never seen in the chromatograms but clearly the His tag-purified MscL under these conditions aggregated much more extensively than did native MscL. This propensity of purified and concentrated MscL-6His to spontaneously aggregate was also observed by analytical ultracentrifugation (*below*). This tendency was never observed in crude extracts of native MscL containing high concentration of endogenous bacterial lipids and other proteins, and was much reduced in dilute solutions of purified MscL-6His stabilized by OG and phospholipids.

Purified MscL-6His in detergent solutions was examined by sedimentation equilibrium. Preliminary runs in nondenaturing detergents (OG, CHAPS, CHAPSO, dodecylmaltoside) showed considerable aggregation as the protein concentration increased as equilibrium was approached. Aggregation of OG solubilized MscL-6His was also noticed while attempting to concentrate the protein in Centricon concentrators and limited the accessible range of initial protein concentrations for the sedimentation equilibrium studies. In some instances where the detergent/protein ratio became quite high, the presence of a low molecular weight species (15–20 kDa) could be detected. In contrast, solubilizing MscL in the presence of phosphatidylcholine at approximately 100:1 molar ratio of OG to lipid considerably reduced the aggregation as well as prevented the appearance of the small component.

Figure 2 shows sedimentation equilibrium data at 2, 4, 6, and 8k rpm at 10°C for a sample with an initial absorbance of 0.248. The data shown contains a non-sedimenting baseline of 0.13 absorbance based on this and higher speed data. In the logarithmic plot the slope at any value of radial position is directly proportional to the weight average molecular weight of all species at that position, assuming they all have the same extinction coefficient per unit mass. Even when the baseline is removed from the data, the logarithmic plots are still curved indicating the presence of two or more species in the sample; this is not surprising given the propensity of MscL to aggregate. The solid curves in the figure are from a global fit of all the data to a model consisting of two noninteracting species and a fixed baseline of 0.13 absorbance. The fit gives a reduced molecular weight (Reynolds & McCaslin, 1985) of 55,200 for the small species and 205,000 for the larger species. A difficulty in fitting these data arises from the formation of the large

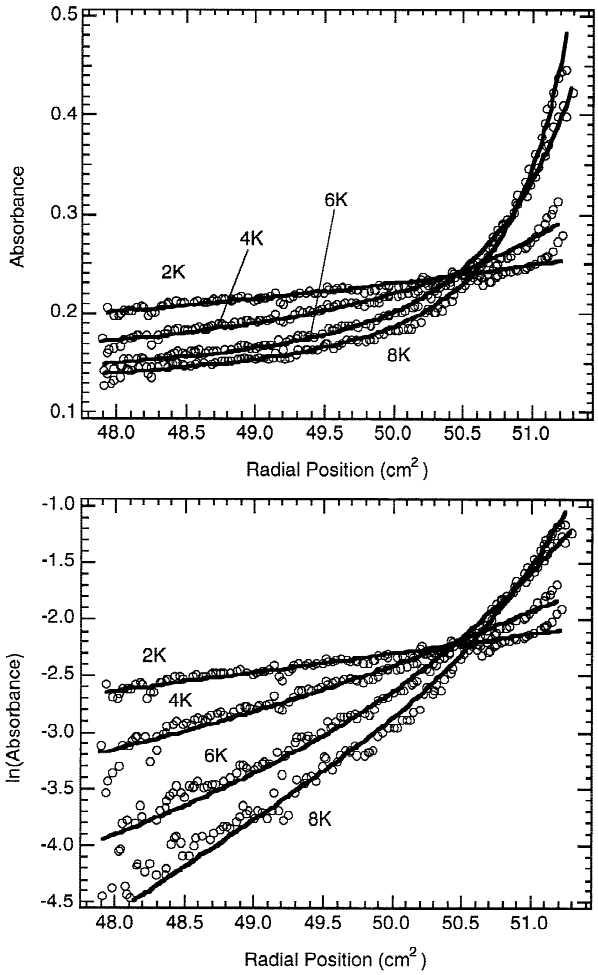

Fig. 2. Sedimentation equilibrium of 6His tag-purified MscL in OG. The initial absorbance at 280 nm was 0.248 in a buffer of 20 mM Na phosphate, 100 mM NaCl and 34 mM OG supplemented with phosphatidylcholine at a ratio of 100:1 OG:lipid. The data shown are for speeds of 2, 4, 6, and 8k rpm and 10°C. The curves on the upper panel show that as the speed increases, the absorbance at small values of radial position approaches a level of approximately 0.13 which was confirmed by high speed depletion. The lower panel is a logarithmic representation of the same data. The solid lines are the result of a global fit of all these data to a model consisting of two noninteracting species and a fixed baseline of 0.13 absorbance (baseline is not subtracted).

aggregates that, in all likelihood, do not represent a single unique species and may vary depending on the initial loading concentration and speed.

55,200 is therefore taken as the reduced molecular weight of the smallest observed species of MscL, in the OG/lipid mixture. As discussed by Tanford et al. (1974), the reduced molecular weight of a detergent-solubilized membrane protein must contain contributions from the bound detergent and lipid as well as the protein. The contribution of lipid to the reduced weight is much smaller than either of the other two and will be neglected. If we assume that there is no detergent in the sedimenting

Table. Protein detergent complexes giving reduced molecular weights of 55,200

Subunits in complex	Mass of protein	Reduced molecular weight	Contributions from:	
			Protein alone	Detergent alone (mol det/mol complex)
1	15800	3800	51400	1250
4	63200	15200	40000	970
5	79000	19000	36200	880
6	94800	22800	32400	800
10	158000	38100	17100	410
15	237000	57100	~0	~0

Specific partial volumes for MscL-6His protein (MW 15,800) and Octylglucoside (MW 292.4) used for calculations were 0.759 and 0.859, respectively. The 1.0 g/ml was used for the density of the buffer and contributions due to lipid (specific partial volume of 0.98) has been neglected.

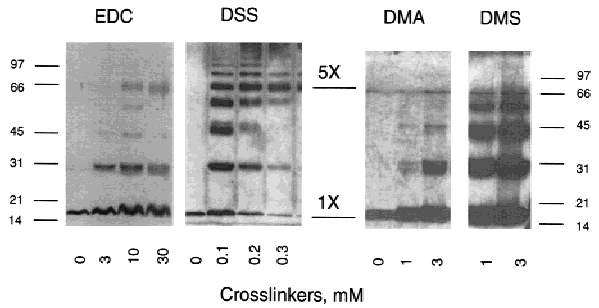

Fig. 3. Crosslinking of purified MscL-6His in OG. The tag-purified MscL protein was transferred into buffers compatable with each crosslinking agent. The agents used was 1-Ethyl-3-(Dimethylaminopropyl) carbodiimide (EDC), disuccinimidyl suberate (DSS), Dimethyl suberimidate (DMS) and Dimethyl adipimidate (DMA) for 1 hr. Crosslinked products were separated by SDS-PAGE and visualized with either Coomassie Blue (left panels) or silver staining (right panels).

particle, a solvent density of 1, and a protein partial specific volume of 0.759 (deduced from amino acid composition), then the observed reduced molecular weight would require a protein species of 229,000 molecular weight or roughly 15 polypeptide chains of 15,781. The presence of detergent in the protein complex reduces the mass of protein required to account for the observed reduced mass, and the Table illustrates possible combinations of MscL subunits and OG molecules that would be consistent with the reduced molecular weight of 55,200. A single MscL subunit would require a binding of about 1,200 molecules of OG/monomer, a value that is highly unlikely in view of the high critical micelle concentration of OG and its small micelle size. Five MscL chains in a single particle would contribute approximately 19,000 to the reduced molecular weight and require 900 detergent molecules per complex or 180 per MscL chain. Assuming 10 MscL chains per particle (equivalent to the association of two pentamers) requires binding of ~400 moles of OG/complex or 40 per MscL chain to account for the reduced molecular weight which given the small size of the octyl chain of OG is likely near the minimum of what might be required to maintain the solubilized protein in solution. On the other hand, the amount of OG/chain with less than 5 or 6 MscL/particle is considerably in excess of the aggregation number of ~84 OG/micelle (Lasser & Elias, 1972) and the binding of such large amounts of OG/chain would seem unlikely.

Sedimentation equilibrium is a thermodynamically rigorous method to measure the molecular weights of molecular assemblies in solution. Unfortunately, the tendency of MscL to aggregate as the protein concentration increases and uncertainty about the amount of detergent bound to the complex have not permitted an unequivocal assessment of the mass of the MscL species as isolated in OG. Nonetheless, under the conditions described here, a significant population of monomeric MscL was not detected and we conclude the MscL exists in the form of aggregates in OG solutions, and likely in native membranes.

Crosslinking In Vitro

Several crosslinking reagents with different reactive groups and arm lengths were applied to detergent purified MscL-6His complexes under nondenaturing conditions. Figure 3 shows crosslinking patterns obtained with the short (4 Å, EDC), intermediate (8.6 Å, DMA) and the long spacer arm (11 Å, DMS and DSS) reagents. Similar gels were developed with both, silver stain for high sensitivity, and with the "linear" stain (Coomassie Blue G250) to enable direct correlation between the density of a band and the amount of a particular product. All crosslinkers generated ladderlike patterns representing crosslinked products with molecular weights consistent with multiples of the monomeric polypeptide. As the concentration of the crosslinker increases, the upper bands become more visible and denser. With EDC and DMS five bands were observed in the ladders the uppermost being at approximately 66 kDa. Increasing the concentration of EDC beyond 30 mM does not increase the number of bands, and silver staining of gels for any of the crosslinkers does not result in visualization of any additional bands. In the case of DMA the largest (fifth) band observed was again approximately 66 kDa, but the fourth band was typically very faint. Since no other proteins are present the 66 kDa band must represent the pentamer of MscL, though its apparent weight is less than the expected 75 kDa. Steele and Nielsen (1978) have observed that oligomers of polypeptides especially larger than a trimer tend to migrate faster in SDS gels and

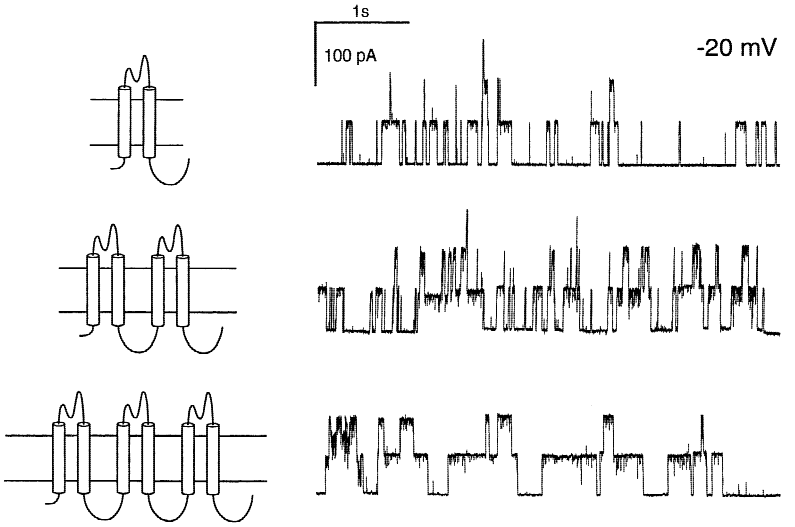

Fig. 4. Functional expression of MscL in double and triple tandems. Two or three linked copies of the MscL ORF were cloned in the pB10a vector and expressed in the PB104 *mscL*$^-$ strain. Giant spheroplasts were generated and surveyed with the patch-clamp technique as described in Materials and Methods. Channels displaying apparently normal 3 nS conductance but slightly different kinetics were recorded in all cases upon suction of 100–180 mm Hg. The number of MscL chains expressed in each case is approximately the same as determined by Western blot (*not shown*). However, the strain expressing WT MscL exhibited many more channels per patch (100–200) compared to the double and triple tandems (1–8 channel/patch). The upper trace is a control recording with the 5-2-2 plasmid coding for the WT MscL, only a small fraction of the channels present are activated.

thus the observed 66 kDa mass is not unreasonable for the pentamer. Thus the upper band of approximately 66 kDa observed when using EDC, DMA, and DMS corresponds to pentamers of MscL.

DSS had the longest spacer arm used in these studies. The ladders obtained exhibited the five bands described above and bands of higher moelcular weight as well. At higher concentrations of DSS, the upper rungs become more intense, whereas lower bands representing monomers, dimers and trimers fade. Importantly, the amount of large molecular weight material remaining at the top of the gel increases with increasing DSS, but this was never observed with the shorter crosslinker EDC. The fifth band, however, remains the most prominent at higher DSS concentrations. Since the gel chromatography and sedimentation equilibrium studies above have shown a propensity for MscL pentamers to aggregate, it is likely that the long arm of DSS permits crosslinking not only within a single pentameric unit but also between pentamers in solution.

Häse and coworkers (1997) performed crosslinking of MscL in intact bacteria and in membrane preparations using a variety of different crosslinking agents. DSS was shown to generate the entire range of products from monomers to pentamers, whereas EDC was less efficient *in situ* and showed monomers and dimers only, even at saturating concentrations. The latter observation led the authors to the conclusion that MscL exists in the membrane predominantly in the form of monomers/dimers and the assembly of active channels could be driven by tension. Similar mechanism of tension-dependent modulation was proposed earlier for alamethicin channels by Opsahl and Webb (1994).

Functional Expression of MscL Subunit Tandems

Native monomeric (E1) and engineered dimeric (E2) and trimeric (E3) forms of MscL were all equally well expressed as evidenced by Western blots following SDS-PAGE (*not shown*). A patch-clamp survey of giant spheroplasts expressing double and triple MscL tandems reveals functional MscL channels (Fig. 4). The unitary conductance of these channels is identical to that of monomeric wild-type channels, although with slightly different kinetic patterns showing slower open-to-closed transitions in E3. The similarity of channel amplitude suggests that the structure of the conducting pathway is likely to be the same as in the case of native monomers. However the number of functional channels observed in tandem-expressing spheroplasts was much lower: we have been able to record 1–4 channels/patch in the double (E2) and 2–8 channels/patch in the triple (E3) tandem as compared to 100–200 channels per patch in spheroplasts expressing the WT monomeric form of MscL (total of 8–10 patches examined for each construct from two independent preparations, with at least three patches exhibiting Po saturation).

The assembly of functional MscL channels from double tandems was previously taken as topological evidence that the MscL polypeptide chain has an even number of transmembrane spans, resulting in the amino and carboxyl termini being on the same side of the membrane. Does the functional expression of MscL from triple tandems require that the channel be composed of a common multiple of two and three, i.e., six subunits? The biochemical data presented above are most consistent with a pentameric stoichiometry for the MscL channel such as was determined from the crystal structure of the MscL homologue of *M. tuberculosis* (Chang et al., 1998). To reconcile these results we must assume that functional pentameric channels can be formed in which the 'extra' subunits from assembling three double or two triple tandems simply do not participate in the channel as suggested by Fig. 7*D*. An extra subunit covalently associated with one pentamer might participate in the forma-

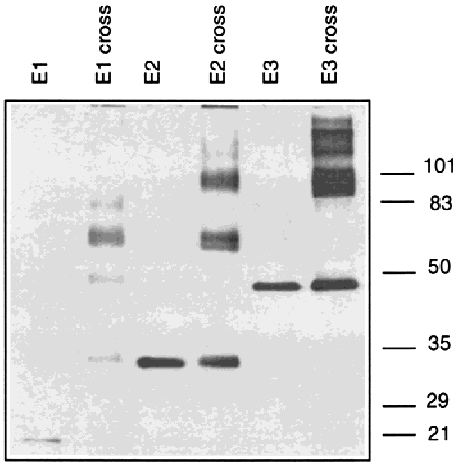

Fig. 5. Crosslinking of expressed WT MscL (E1) and of double (E2) and triple (E3) MscL tandems *in situ.* Membranes containing the MscL variants were allowed to react with disuccinimidyl suberate (DSS) for 30 min. DSS concentration was 1 mM for E1 and 0.1 mM for both E2 and E3. (*B*). E1 exhibits a whole range of intermediate products with 5× as a maximal degree of crosslinking; E2 has one 4× intermediate with 6× multimers as the most represented product. E3 crosslinks immediately into hexamers and also shows larger products.

tion of a neighboring channel (Fig. 7*E*) and could potentially lead to formation of chains of complexes (concatamers). Generation of subunit tandems has been an informative approach in studies of several types of multimeric channels. Experiments with Shaker (McCormack et al., 1992) and other tetrameric potassium channels (Liman, Tytgat & Hess, 1992) suggested that the tandem linkage does not always guarantee the stoichiometry of the expressed channel complexes such that some of the subunits linked in one polypeptide chain may not be functionally involved in the complex.

Covalent Crosslinking In Situ

Native membranes were prepared from strains expressing either wild-type (single polypeptide chain), double or triple tandems of MscL and treated with the crosslinking agent DSS. The products were separated by gel electrophoresis and visualized as Western Blots. As shown in Fig. 5, the largest species for WT MscL was a pentamer and all intermediate oligomers were evident. The crosslinked double tandem shows bands equivalent to four and six MscL chains (two and three double tandems linked together). The triple tandem exhibits a major crosslinked product equivalent in mass to six MscL chains (two trimers linked) and a number of less well-defined bands of even higher mass. Neither of the other MscL constructs exhibit bands not readily attributable to oligomers of MscL; thus these high molecular weight bands must represent crosslinked MscL triples and not cross-linking of neighboring non-MscL polypeptides to MscL with subsequent visualization by the anti-MscL antibody.

As evident in Fig. 3, multiples larger than the pentamer were observed when DSS was used to crosslink detergent purified 6His-tagged MscL. In contrast, we observed no visible products larger than pentamers upon crosslinking native MscL or MscL-6His *in situ.* When DMS was used as a crosslinker in the detergent purified system, aggregates higher than the pentamer were not observed, but were observed in crosslinking the membranes containing triple tandems of MscL (*not shown*). Thus aggregates larger than the pentamer in these membrane studies on tandems seem to arise for reasons distinct from those observed in the detergent solution. Since the membranes contain similar amounts of protein regardless of the nature of the MscL construct, and very different numbers of functional channels, the crosslinking results could reflect nonspecific, nonfunctional aggregation of MscL tandems within the membranes. As discussed earlier and illustrated in Fig. 7, the "extra" subunits brought into the channel by the tandem constructs could lead to interactions among channels and formation of concatamers which could also lead to aggregates much larger than the pentamer in crosslinking studies.

Comparison of Molecular Sizes of MscL, Complexes Assembled from Monomers and Double Tandems

The complexes assembled from double tandems must have an even number of MscL subunits and the largest product observed in crosslinking studies demonstrated the equivalent of six MscL chains as the largest product (Fig. 5). This suggests that the preferential assembly of MscL made of double tandems is hexameric. The dramatic disparity in number of functional channels formed as well as differences in crosslinking patterns suggest that the monomers and dimers may assemble differently. Possible differences in channels assembled from monomers and double tandems were explored by mixing crude membrane preparations from expressor strains for each, solubilizing the mixture with 3% OG, and running it through a calibrated Superose 6 HR column. The presence of monomeric or dimeric forms of MscL was determined by Western blots of small individual fractions (Fig. 6). The elution profile of native MscL shows a single sharp peak at 65–70 kDa MW, consistent with our previous observation (*see* Fig. 1). Another peak eluted earlier than the complexes formed from monomeric MscL (around 90 kDa) was composed only of the double tandems. A slight "smearing" of dimeric MscL across several fractions (*not shown*) also indicates that complexes made of dimers are heterogeneous in size. The

fact that the native MscL elutes as a particle slightly lighter than the pentameric structure predicts (75 kDa) can be explained by hydrophobic interaction of MscL with the Superose matrix, the possibility noted in the manufacturer's manual for the column. Irrespective of the absolute values, the shift between the peaks of native MscL and "forced" hexamers assembled from tandems roughly corresponds to the contribution of one subunit. Since the shift is relatively small, running the mixed sample through the column was the most reliable way to detect it as opposed to separate runs with two different complexes. These results clearly demonstrate that complexes formed by tandems are larger in either mass and/or size and are more heterogeneous than those formed from native MscL. We never observed smearing of native MscL in the column upon mixing with extracted tandem complexes, which suggests that the complexes do not exchange with subunits.

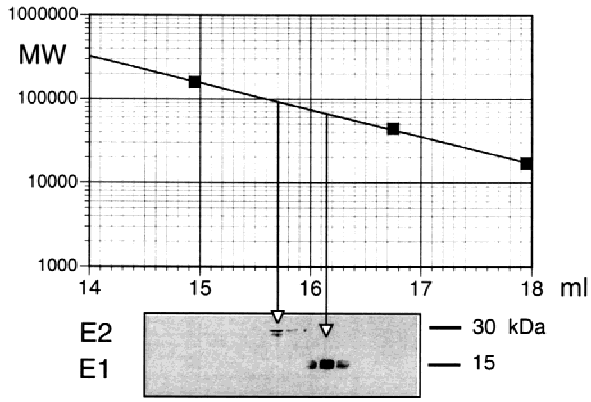

Fig. 6. Gel chromatography of OG solubilized MscL complexes assembled from WT monomers (E1) and double tandems (E2). Membranes from E1 and E2 expressing strains were mixed and extracted with 3% octylglucoside. 0.1 ml of the extract was loaded onto a calibrated Superose 6 column. Peak positions of E1 and E2 were determined on a Western blot from individual 150 μl fractions. E1 elutes later indicating smaller size of the native MscL complex.

Discussion

In the present work we have applied a number of approaches to examine the stoichiometry and stability of the MscL channel. A fully rigorous determination of the mass of protein associated with detergent-solubilized MscL by sedimentation equilibrium has been precluded by the tendency of the His-tagged MscL to aggregate with increasing protein concentration and by uncertainties in the amount of bound detergent. The data have however, provided limits as to the mass of the smallest species present in detergent solution. This species is not monomeric which is consistent with size-exclusion chromatography results. Moreover, the chromatography data also indicated that native MscL in membrane extracts exists as a population of uniform particles larger than the monomer, i.e., preformed complexes, which practically excludes the hypothesis that MscL assembly is a dynamic equilibrium (Häse et al., 1997).

Previously, we proposed a hexameric model of MscL channel stoichiometry based on the use of a single type of crosslinking agent (DSS) and this seemed to be supported by the observation that genetically engineered double and triple tandems of MscL could form functional channels. Recent crystallographic data, however, revealed a homopentameric organization for the MscL homologue from *Mycobacterium tuberculosis.* Crosslinked products observed in the present study using a variety of crosslinkers (EDC, DMA, and DMS) are more compatible with a pentameric organization of *E. coli* MscL (Fig. 3) as well.

The patterns observed with DSS seem to be an exception in that oligomers higher than the pentamer were seen in detergent-purified His-tagged MscL and larger than "hexamers" assembled from the double and triple tandem construct. Since DSS has the longest crosslinking spacer (11.4 Å), and in the case of the detergent solubilized MscL, gel exclusion chromatography and sedimentation equilibrium clearly demonstrate the presence of species larger than a pentamer, it is possible that the long reach of DSS and the chemistry of its NHS-ester groups permits the formation of crosslinks between MscL subunits in nearby channel assemblies. The spacer in DMS is only 0.4 Å shorter than that of DSS and since both are reactive towards amino groups, one would reasonably expect it to generate a pattern similar to DSS. Yet products larger than a pentamer were never seen with DMS as the crosslinker (Fig. 3), suggesting that the distinct pattern of products observed with DSS is due to more than just the length of the spacer and likely reflects a difference in chemical reactivity as well as accessibility. The crosslinking results for the engineered tandems in membranes could reflect merely nonspecific aggregation of functionally incompetent MscL tandems in the membrane and/or be due to channel interactions (concatamerization) via the "extra" subunits associated with each functional pentameric channel.

Experiments with tandems of genetically linked MscL subunits revealed a number of differences in the efficiency of functional channel assembly, maximal size of crosslinked product, and behavior of solubilized complexes in a size-exclusion column as compared to the native MscL. MscL complexes formed from double tandems which were capable of forming functional channels must have an even number of subunits though not all of the subunits need to be part of a functional channel (Fig. 7). Crosslinking patterns for the tandems in membranes (Fig. 5) strongly suggest the equivalent of six MscL chains or three double tandems are the fundamental membrane assembly of MscL. Gel filtration experiments

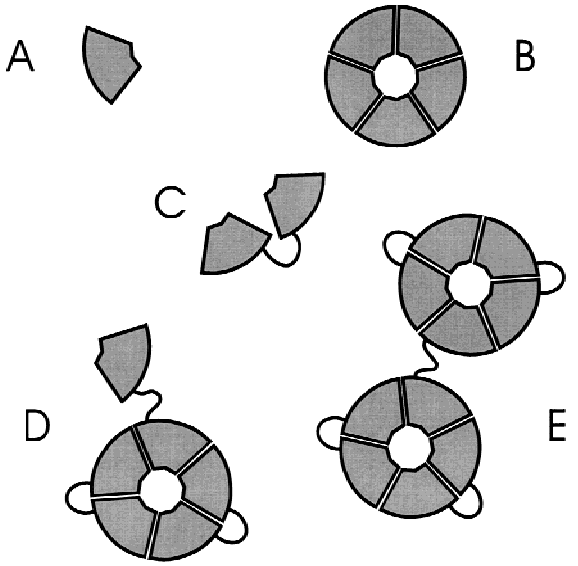

Fig. 7. A possible scheme for assembly of pentameric MscL channels from single subunits and genetically linked double tandems. (*A*) single subunit; (*B*) native pentameric channel; (*C*) double tandem subunit; (*D*) "hexamer" formed from three double tandems with a pentameric channel as the core; (*E*) connection of two MscL channels formed from double tandems using the "non-channel" subunit of one. Interactions such as these in *E* could lead to larger concatamers.

on a mixture of solubilized membranes containing double tandem MscL and membranes containing wild-type MscL, clearly show that the wild-type MscL elutes later than the double tandem. Therefore the solubilized form of wild-type MscL is smaller than the solubilized form from the tandem and is a pentamer rather than a hexamer.

The double and triple tandems, although expressed at comparable levels of total protein as the native monomer, produce relatively small numbers of functional channels. The channels are characterized by the same 3 nS unitary conductance as the native MscL; thus, the open conformation of the channels formed from these constructs consisting of two or three MscL chains, covalently linked together through genetic manipulation, appears to be the same as that of the native channel assembled from five single monomeric chains. We assume that a functional channel contains only five MscL chains independent of the number of subunit repeats in the polypeptide precursor (Fig. 7). Similar precursor-independent assembly has been reported for tetrameric Shaker (McCormack et al., 1992), and mammalian KV1.1 (Liman et al., 1992) channels. The presence of the "extra" subunits presumably not incorporated into the channel structure proper may account for altered gating kinetics of channels recorded from the triple tandem preparation (Fig. 4). The covalent structure of the double and triple tandems will of necessity bring at least one "extra" subunit into the vicinity of each pentameric channel which might then be incorporated into neighboring pentameric channels and lead to the formation of chains of connected channels, or nonspecific complexes, which are nonfunctional. The latter could account for the low numbers of functional channels observed in the patch-clamping experiments.

With regard to molecular structure, crystallography provides the most detailed structural information, although it is not always completely artifact-free. In some cases crystallization conditions may affect the conformation and even the stoichiometry of multisubunit assembly. For instance, X-ray crystal structure of staphylococcal alpha-hemolysin obtained in the absence of lipids shows heptameric assembly (Song et al., 1996), whereas the same toxin clearly forms hexamers in supported phospholipid bilayers as revealed by atomic force microscopy (Czaijkowsky, Sheng & Szao, 1998). The latter probably better corresponds to the membrane-inserted state of this pore-forming toxin. The crystallization of the MscL homologue from *M. tuberculosis* was done at low pH in the presence of heavy metals (Chang et al., 1998), which stabilized the crystals but could potentially bring some distortion to the native conformation. The data presented here are in agreement with the crystallography data with respect to the stoichiometry of the MscL channel and support the conclusion that the family of MscL-like channels are homopentamers.

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