A Hexameric Transmembrane Pore Revealed by Two-dimensional Crystallization of the Large Mechanosensitive Ion Channel (MscL) of *Escherichia coli*

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We have established a reconstitution method of the detergent-solubilized recombinant large mechanosensitive ion channel of *Escherichia coli* (MscL) that yielded two-dimensional crystals. For that purpose, we have developed a new protocol using Triton X-100 to solubilize and purify the MscL protein. This protocol not only allowed an increase in the yield of the protein but also made it possible to obtain a homogeneous delipidated and reproducible preparation of the purified protein. When examined by the patch-clamp method MscL channels were found to be fully functional, exhibiting characteristic conductance and activation by pressure. For electron crystallography the homogeneous Triton X-100-purified recombinant MscL was further reconstituted at low lipid-to-protein ratios using Bio-Beads SM2 to remove the detergent. Two-dimensional crystals, exhibiting a p6 plane group symmetry, have been produced and examined by negative stain electron microscopy. Image processing of selected micrographs yielded a projection map at 15-Å resolution that provided the first explicit structural information about the molecular boundary and homohexameric organization of the MscL channels in the membrane bilayer.

Models of the folding topology of membrane channels, primarily based on hydrophy plot analysis of amino acid sequences, have guided biochemical, biophysical, and molecular biological experimental approaches toward better understanding of the sequence-structure determinism of these macromolecules. Though powerful, these approaches do not reveal what structural elements constitute and control the selective permeability of ion channels and are limited in scope in the absence of high resolution structures determined by either two-dimensional or three-dimensional crystallography. Porins (1, 2), whose atomic structure has also been elucidated in X-ray studies (3), and the nicotinic acetylcholine receptor channel (4, 5) are to date among the best characterized membrane pores with their structure determined by electron crystallography. Although two-dimensional crystals of membrane proteins have been extremely useful for determining their detailed three-dimensional structures the principal obstacle in the way of progress toward determination of tertiary structure of other ion channels has been the difficulty to produce high amounts of pure channel proteins required for electron crystallography. In this context, the large mechanosensitive ion channel (MscL) of *Escherichia coli* presents an excellent candidate for two-dimensional electron crystallography because of (i) the bacterial source providing a relatively abundant of the MscL protein and (ii) the sturdiness of this channel protein that allows for its easy overproduction, purification, and liposome reconstitution required for the two-dimensional crystallization.

Despite its obvious biological significance, there is a considerable lack of knowledge of molecular structures and mechanisms underlying the mechanosensitivity of mechanosensitive (MS) ion channels. This has been largely a result of the lack of both structural information and specific pharmacological agents for this ubiquitous and diverse family of ion channels (6–12). However, the cloning of the MscL, the large conductance MS channel of *E. coli* (13, 14), has provided an MS channel protein that to date has been the only MS channel available for structure and function studies (12, 15–21). The mscL gene encodes a small 15-kDa protein of 136 amino acid residues with two transmembrane segments connected by a periplasmic loop, which appears to be a common structural motif of a new family of structurally related ion channels having two membrane-spanning α-helices (22). The amino and carboxyl termini are located in the cytoplasm, and the functional channel appears to be a homohexamer as suggested by cross-linking studies (18, 21). The channel is gated by mechanical force transmitted via membrane lipid bilayer alone because it remained functional upon reconstitution in pure lipid bilayer (15). How this is achieved is yet unknown, although a recently proposed electromechanical coupling model of MscL gating (23) may account for the channel mechanosensitivity at the molecular level.

In the present study, we describe a method that allowed a successful generation of two-dimensional crystalline arrays of MscL, whose crystallographic analysis yielded a projection map at 15-Å resolution. The map showed the molecular boundary and hexameric organization of the MscL channels in the membrane bilayer, thus making MscL the first MS ion channel with its gross tertiary structure revealed.

EXPERIMENTAL PROCEDURES

**Protein Purification—E. coli** strain AW737-KO, carrying the plasmid pGEX1.1 and encoding the glutathione S-transferase-MscL fusion protein, was grown as described previously (15). The cells were harvested, resuspended in 30 ml of 50 mM NaH2PO4, pH 7.6, 100 mM NaCl, 5%
sucrose, 2 mM MgSO₄, DNase (20 μg/ml) and passed two times through a French press at 8,000 p.s.i. The cell suspension was centrifuged for 45 min at 4,300 × g to eliminate cell debris and inclusion bodies. The supernatant was incubated with 1% Triton X-100 and added to 1 ml of glutathione-Sepharose 4B beads for 3 h at room temperature. The beads were then washed four times by centrifugation using a desktop centrifuge for 5 min in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.2) containing 0.5% Triton X-100. After the last centrifugation, the beads were resuspended in 100 mM NaCl, 10 mM Tris, pH 8.0, 0.2% Triton X-100. Thrombin was added to a final concentration of ~1 unit/μg of protein and incubated for 3 h at room temperature. The beads were transferred to a small column (10 × 30 mm) and washed two times with 250 μl of 10 mM Tris, pH 8.0, 100 mM NaCl, 0.2% Triton X-100. Protein samples were analyzed by 15% SDS-polyacrylamide gel electrophoresis.

**Liposome Reconstitution and Patch-clamp Recording**—The Mscl protein purified with Triton X-100 was incubated with azolectin liposomes at the protein-to-lipid ratios of 1:6,000 or 1:4,000 (w/w) for 30 min at room temperature before the addition of Bio-Beads SM-2 (Bio-Rad) at a concentration of 80 mg of Bio-Beads per ml of protein solution in 0.2% Triton X-100. After 4 h Bio-Beads were discarded, and the suspension was centrifuged for 25 min at 90,000 rpm. The pellet was resuspended in 20 μl of HEPES-KOH pH 7.0, and aliquots were subjected to a dehydration/rehydration to obtain giant proteoliposomes as described previously (15). The giant proteoliposomes, collapsed in the recording chamber containing 200 mM KCl, 40 mM MgCl₂, 10 mM Hepes, pH 7.0, were examined for mechanosensitive currents using the standard patch-clamp technique (15, 24, 25).

**Two-dimensional Crystalization and Electron Microscopy**—Purified Mscl was resuspended in 100 mM KCl, 20 mM Tris, pH 8.0, 0.1% Triton X-100 and supplemented with *E. coli* lipids. The protein concentration was adjusted to 1 mg/ml, and the lipid-to-protein ratio to 0.45 w/w. The samples were incubated for 1 h in a cold room with gentle stirring and were subsequently treated with Bio-Beads SM2 according to the batch procedure as described previously (26). Typically, the detergent removal was performed in the cold room by adding first 5 mg of Bio-Beads to 50 μl of the reconstitution mixtures for a period of 4 h, followed by a second addition of 5 mg of Bio-Beads for about 1 h to ensure the complete detergent removal. The reconstituted material was then separated from the Bio-Beads with a micropipette and incubated overnight at 4 °C before carrying out three cycles of freezing (−190 °C) and thawing (25 °C). Aliquots were taken daily and examined by electron microscopy of the reconstituted material.

**RESULTS**

**Solubilization and Purification of Mscl in Triton X-100**—Initial attempts to crystallize Mscl purified with octyl glucoside have been unsuccessful. A mixture of protein aggregates and amorphous pure lipid structures was observed after removal of the detergent, indicating non-mixing of lipids and proteins in the original micellar solutions and/or protein aggregation at low lipid content in the presence of this detergent. Because two-dimensional crystallization of membrane proteins has been shown to drastically depend upon the nature of the detergent, we focused on the development of a new method using Triton X-100 instead of octyl glucoside for the Mscl solubilization.

As the first step during the extraction procedure, we used the French press to eliminate the inclusion bodies that might have contained non-functional and/or misfolded Mscl proteins. Four consecutive washes of glutathione-Sepharose columns in the presence of Triton X-100 ensured an almost complete delipidation of Mscl (approximately 10% lipid/β protein). The final step was to minimize the volume of the solutions during the enzymatic cleavage. This ensured a high protein concentration (0.7–1.2 mg/ml) and thus made unnecessary the concentration of the protein by Amicon filters that usually led to unknown final detergent concentrations. The amount of the pure protein obtained with Triton X-100 was approximately 1 mg of Mscl/L1 liter of cell culture, a yield larger by a factor of 1.3–2 when compared with that obtained using octyl glucoside.

After 5 h of incubation with the Bio-Beads ensuring complete

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**Fig. 1. Purity and activity of Mscl purified using Triton X-100.** A, SDS-polyacrylamide gel electrophoresis of purified Mscl proteins. Lanes: 1, molecular mass markers; 2, Mscl purified with octyl glucoside; 3, Mscl purified using Triton X-100. The same amount of the Mscl protein was loaded in both lanes. B, pressure sensitivity of the Mscl protein. The traces are 2-s recordings at a pipette potential of −20 mV and negative pressures applied to the interior of the pipette, as indicated. C denotes the closed state and O denotes the open state of n number of channels.

SDS-polyacrylamide gel electrophoresis analysis of the Mscl proteins purified with Triton X-100 revealed a large and diffuse band around 17 kDa, similar to the pattern observed with Mscl purified using octyl glucoside (Fig. 1A). **Patch-clamp Analysis of the Reconstituted Mscl**—We have also functionally examined the channel activity of Mscl purified using Triton X-100. When examined by the patch clamp the reconstituted proteins were activated by negative pressure (suction) in a way similar to what was reported previously (15) (Fig. 1B). The channel sensitivity to pressure in the particular patch was estimated to be 5.9 mm Hg per exponential-fold change in open probability, and the negative pressure required to induce half-maximal activation of Mscl was 40 mm Hg at a pipette potential of −20 mV. The single channel conductance was 3.5 ± 0.2 nS (n = 3) (data not shown) (15). Furthermore, between 86 and 100% of the patches examined (n = 20) were found to contain functional Mscl proteins. A similar result of 83% active patches (n = 41) was obtained with Mscl purified using octyl glucoside and reconstituted into liposomes at the same protein-to-lipid ratio. These results are comparable with those previously reported (15, 17).

**Two-dimensional Crystalization of Mscl**—Two-dimensional crystals were obtained at low lipid-to-protein ratios (0.3–0.6, w/w) by mixing the purified Triton X-100-solubilized Mscl with small amounts of *E. coli* lipids. The detergent was then removed from these micellar solutions by hydrophobic adsorption onto Bio-Beads SM2. Electron microscopy of samples obtained after the treatment with the Bio-Beads revealed vesicular crystals. The order and appearance of crystalline arrays were optimal at a lipid-to-protein ratio of 0.45 w/w. Lower ratios decreased the number of vesicular crystals at the expense of lipid-protein aggregates, whereas higher ratios lead to proteoliposome formation with a decreasing degree of crystallinity.
Two-dimensional Crystallization of MscL

In this study we described a method that for the first time made possible a two-dimensional crystallization of a mechano-sensitive channel providing information about its structure at the resolution of 15 Å. We believe that the new protocol of the MscL solubilization and purification using Triton X-100 and the strategy of two-dimensional crystallization employing BioBeads SM2 as a detergent-removing agent (26) has been essential for developing this structural approach.

Previously, MscL has been purified using octyl glucoside as a solubilizing agent (13, 15–17). However, octyl glucoside was found not to be useful for the two-dimensional crystallization procedure because of the larger variability in the purity of the final MscL protein fraction purified using this detergent as

detergent removal at 4 °C, electron microscopy revealed vesicles of approximately 50–75 nm in diameter with densely packed particles and with about 50% of them already crystalline (data not shown). The formed crystals did not grow upon further incubation and were very stable, existing for weeks. After several freeze-thaw cycles, some of the crystalline vesicles fused to give larger vesicles of about 200 nm in diameter with crystalline arrays covering all the vesicles (Fig. 2A).

Despite their small size, optical diffraction of the negatively stained MscL crystals showed strong reflections on a hexagonal lattice with resolution up to about 30 Å. We selected single layered two-dimensional crystals corresponding to split-open vesicles. Fig. 2, A and B, shows electron micrograph and Fourier transform of such a single layered two-dimensional crystal. Interestingly, the filtered image of the two-dimensional crystal (Fig. 2C) clearly shows a hexagonal packing of cylindrical particles, which are characterized by a central depression.

**Image Analysis and Projection Map**—We selected the 20 best micrographs of crystals exhibiting the appearance and optical density of single layers. The unit cell parameters were calculated as: $a = b = 57$ Å and $\gamma = 120$ Å. When the micrographs were processed to correct for crystal distortion according to the procedure of Henderson et al. (27), statistical analysis of the data indicated significant structural information up to a resolution of about 15 Å. Examination of the phase residuals using the ALLSPACE program indicated a $p_6$ plane group (28). We merged 14 images to produce a projection map at 15-Å resolution (see IQ plot in Fig. 3B). The striking resemblance of the maps without (Fig. 3A) and with $p_6$ symmetry applied (Fig. 3C) showed that the MscL channel molecules in the unit cell were indeed related by a 6-fold symmetry.

The projection map clearly delineates the contour of the channel molecules (the molecular boundary of the particles). This map shows that the molecules consist of hexagonal particles, with an outer diameter between 42 and 48 Å. The stain-excluding regions are arranged in a ring with a radial width of approximately 11–15 Å, which surrounds a central depression of approximately 18 Å in diameter. Within each ring, there are six round regions of density that are clearly resolved and demonstrate a homohexameric organization of the MscL channels. Because negative staining revealed the overall topography of the surfaces exposed to the aqueous medium, it is likely that a significant fraction of each monomer projects out of the hydrophobic core of the membrane. The predicted external domains of the MscL monomer (S1, S2, S3, and C terminus) (13) probably contribute to these densities.

**DISCUSSION**

In this study we described a method that for the first time made possible a two-dimensional crystallization of a mechano-
Two-dimensional Crystallization of MscL

compared with the purity of MscL extracted by Triton X-100. One of the possible explanations includes delipidation as the crucial step, because delipidation of the protein could lead to protein aggregation and/or denaturation in the presence of octyl glucoside but not Triton X-100. Also, protein aggregation may occur during two-dimensional crystallization because of inefficient mixing of lipids and proteins that could lead to lipid-rich micelles and protein-rich micelles and/or preferential octyl glucoside removal from one type of micelles.

The Bio-Beads were recently reported to provide an efficient alternative method to conventional dialysis for removal of detergents and for obtaining two-dimensional crystalline arrays of membrane proteins (26). This method has been successfully applied for the generation of crystals of different classes of membrane proteins solubilized in different detergents (26, 29, 30). Also, as demonstrated in this study, it was very efficient in producing two-dimensional crystals of MscL.

The patch-clamp analysis of the MscL proteins reconstituted by the same method as the one used for the two-dimensional crystallization demonstrated that the MscL channel remained fully functional in terms of its pressure sensitivity as well as conductive properties. These results strongly suggest that the two-dimensional MscL protein crystals represent the oligomeric tertiary structure of the channel that most likely reflects its functional assembly in the native bacterial membrane. Several independent studies suggested that the functional MscL channel is a homohexamer (18, 20, 21). Our data obtained by electron crystallography showed that the MscL monomers are arranged in almost equivalent positions conferring upon the structure a high degree of hexagonal symmetry and demonstrated clearly that the MscL does form homohexameric ion channels.

The other prominent feature of the projection map is the central depression domain of approximately 18 Å in the center of the channel molecule. A first interpretation would be to consider a channel model in which hexameric organization produces an aqueous pore in the middle of the channel that should be accessible from both sides of the membrane. However, from permeation studies using large organic molecules, the size of the open channel pore of MscL was estimated to be approximately 35–40 Å in diameter (31), i.e. much larger than the 18 Å measured from the projection map. In addition, because there is no reason to believe that the bilayer was stretched in our MscL crystallization conditions, the hexamer most likely depicts the closed channel. Thus, a second more plausible interpretation would be to assume that the projection map represents the channel in the closed state. Indeed, taking into account that the negative stain does not penetrate into bilayer, only the extramembranous domains can be visualized. Thus, the depression in the center of the hexamer may be filled by the transmembrane helices kinked or oriented at an oblique angle with respect to the membrane plane and not detectable in our experimental conditions. Because no proline residues are present in the transmembrane regions of MscL, possible closure of the channel by kinked α-helices could involve a leucine ring as reported for the acetylcholine receptor channel (5); according to this hypothesis, there are two leucines in the M1 helix and one leucine in the M2 helix of each MscL monomer.

This interpretation has an important consequence for the closed-to-open conformation of the MscL channel molecule upon stretching the membrane. Indeed, in the open configuration, the channel pore is 35–40 Å in diameter. Consequently, the minimal calculated external diameter of the hexameric channel should be about 50–60 Å (31). Compared with a diameter of 42–48 Å measured on the projection map for the closed channel, this indicates a substantial conformational change in different domains of the channel hexamer. It is known that proteins undergo substantial internal motions, ranging from side chain realignments on a picosecond time scale to large transitions in folding occurring over several microseconds. Recent mutagenesis studies have revealed major internal motions during the gating of Na⁺ channels: upon depolarization, the S4 voltage sensors move 4.5–11 Å within 1 ms (32).

At present, the issue of the precise molecular organization of secondary structural elements forming the channel with a pore in the center of the hexamer cannot be resolved until higher resolution maps are calculated. Frozen-hydrated MscL crystals should contain additional information on the channel protein structures buried within the hydrophobic region of the phospholipid bilayer in which the stain cannot penetrate. This, however, will be the subject of further crystallographic studies aiming toward improving the quality of the two-dimensional MscL crystals that will allow us to decipher the structure of this MS channel at the atomic level. With the current convergence of conceptual and technical breakthroughs, we stand at the threshold of an exciting phase in the structural analysis of this membrane protein.

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