A Tetrameric Porin Limits the Cell Wall Permeability of Mycobacterium smegmatis*

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Mycobacteria protect themselves with an outer lipid bilayer, which is the thickest biological membrane hitherto known and has an exceptionally low permeability rendering mycobacteria intrinsically resistant against many antibiotics. Pore proteins mediate the diffusion of hydrophilic nutrients across this membrane. Electron microscopy revealed that the outer membrane of Mycobacterium smegmatis contained about 1000 protein pores per μm², which are about 50-fold fewer pores per μm² than in Gram-negative bacteria. The projection structure of the major porin MspA of M. smegmatis was determined at 17 Å resolution. MspA forms a cone-like tetrameric complex of 10 nm in length with a single central pore. Thus, MspA is drastically different from the trimeric porins of Gram-negative bacteria and represents a new class of channel proteins. The formation of MspA micelles indicated that the ends of MspA have different hydrophobicities. Oriented insertion of MspA into membranes was demonstrated in lipid bilayer experiments, which revealed a strongly asymmetrical voltage gating of MspA channels at ~30 mV. The length of MspA is sufficient to span the outer membrane and contributes in combination with the tapering end of the pore and the low number of pores to the low permeability of the cell wall of M. smegmatis for hydrophilic compounds.

About two million people die each year from tuberculosis (TB). This number qualifies TB as the leading agent of death due to a single infectious disease (1). Treatment of TB caused by non-resistant strains of Mycobacterium tuberculosis is effective but is based on a regimen of up to four drugs over a period of six months (2). The major problem in TB therapy is the slow uptake of drugs across the mycobacterial cell wall, which mounts a formidable permeability barrier toward diffusion of hydrophobic and hydrophilic compounds (3). The diffusion of hydrophilic compounds across the mycobacterial cell wall is mediated by water-filled channel proteins (4). The transport function of these channel proteins is similar to that of the porins of Gram-negative bacteria, but the porin pathway of mycobacteria is at least 1000-fold less efficient than that of Escherichia coli (5, 6). It is assumed that the combination of low cell wall permeability with the action of detoxifying proteins such as degrading enzymes or efflux pumps is responsible for the intrinsic resistance of mycobacteria to many antibiotics such as penicillins, cephalosporins, and tetracyclines (7). However, it is not known why the porin pathway in mycobacteria is so inefficient.

Two integral proteins with transport properties have been identified in mycobacterial cell walls: OmpATb from M. tuberculosis, which has channel activity in vitro but unexplored physiological functions (8), and MspA, which was first discovered in chloroform-methanol extracts from Mycobacterium smegmatis as an oligomeric channel protein composed of 20 kDa subunits (9). Enzyme-linked immunosorbent assays and immunogold labeling experiments demonstrated that MspA is localized in the cell wall (6). Uptake of glucose by an mspA deletion mutant was 4-fold slower compared with the wild type indicating that MspA is the major porin of M. smegmatis (6). MspA is the prototype of a family of four very similar porins of M. smegmatis, which did not show any homology to any other known protein (6). Up to now, MspA is the only mycobacterial porin, which can be purified in milligram quantities (10) and is, therefore, amenable to structural investigations.

The structure of mycobacterial porins would be of paramount importance both for understanding the nutrient transport across the mycobacterial cell wall and for treatment of TB with hydrophilic drugs but is also likely to be of fundamental scientific interest because porins reside in an asymmetric outer membrane with unique properties. (i) The membrane has a very low fluidity and does not melt up to 70 °C in contrast to cytoplasmic membranes of other mesophilic organisms, which begin to melt at 20 °C (11), (ii) it is about 10 nm thick, exceeding the thickness of all other known membranes by about 2.5-fold (3), (iii) it provides a very hydrophobic cell surface, which causes the bacteria to clump in an hydrophobic environment, and (iv) its fluidity decreases toward the periplasmatic side of the membrane (12) in contrast to that of the outer membrane of Gram-negative bacteria.

Here, we present the first structural analysis of a mycobacterial porin. The MspA complex is composed of four subunits and forms a single pore of extraordinary length. A hydrophobicity gradient along the surface of the MspA pore leads to an oriented membrane insertion in planar lipid bilayers. The structural properties of MspA and the low number of porins contribute to the low permeability of the cell wall of M. smegmatis compared with E. coli.

MATERIALS AND METHODS

Purification of MspA—MspA was purified from M. smegmatis by selective extraction at 100 °C using 9.5% n-octyl-polyoxyethylene (octyl-POE) as a detergent and a two-step chromatographic purification procedure as described (10). MspA eluted from the anion exchange column

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‡ The abbreviations used are: TB, tuberculosis; POE, polyoxyethylene; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMPC, dimyristoyl phosphatidylcholine.

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at 0.57 M NaCl and was separated from another porin in the extracts, MspC, which eluted at 1.3 M NaCl as described (13). Purified MspA was stored in a 25-mM sodium phosphate buffer at pH 7.5 containing 0.5% octyl-POE (NaP-POE buffer). Subpicogram amounts of purified MspA showed a high channel-forming activity in planar lipid bilayer experiments, which were done as described (14).

Cross-linking Experiments—Glutardialdehyde, the most commonly used cross-linking reagent, could not be used because MspA contains only a single lysine residue. Therefore, water-soluble carbodiimides were employed to cross-link carboxyl groups of MspA (14). To obtain a maximal yield of cross-linked protein the molar ratio of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and cystamine to MspA was optimized. In the final reaction, 1 μg of MspA in 100 μl (0.13 μM) was incubated with 76 μM EDAC and 0.95 μM cystamine for 90 min at room temperature. The reaction was buffered with 25 mM sodium phosphate at pH 7.0. The sample was dialyzed for 12 h against 400 ml of 25 mM sodium phosphate (pH 7.0). Four-fifths of the sample containing 800 ng of MspA was denatured by heating in 80% dimethyl sulfoxide at 37°C for 30 min and precipitated with acetone (9) before gel electrophoresis, whereas one-fifth of the sample containing 200 ng of MspA was directly loaded on the gel. Gel electrophoresis and staining with methylene blue were performed in 37°C. The electrophoresis, whereas one-fifth of the sample containing 200 ng of MspA was directly loaded on the gel. Gel electrophoresis and staining with silver was done as described (10).

Analytical Gel Filtration—The molecular mass of MspA was determined by analytical gel filtration. A silica-based (G3000SW, Tosoh Biosep) and dextran-based material (Superdex 200, Pharmacia) were used for gel filtration to detect errors resulting from unspecific interactions with MspA. Chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), phosphorylase b (97.4 kDa), and aldolase (158 kDa) were taken for calibration.

Electron Microscopy and Single Particle Analysis—Isolated and solubilized MspA was either dialyzed to remove the detergent or reconstituted into lipid vesicles prior to inspection in the electron microscope. MspA was dialyzed against water for at least 24 h, diluted to a protein concentration of ~0.1 mg/ml and applied to carbon-coated copper grids made hydrophilic by glow discharge. MspA samples and cell wall fragments were negatively stained with a solution of 0.2% unbuffered uranyl acetate and inspected in a Philips EM-420 or CM12 transmission electron microscope at a primary magnification of 35,000-fold. For documentation electron micrographs were taken and scanned using the CCD Scanner Flextight (Imacon, Copenhagen Denmark). Reconstitution of solubilized MspA in lipid membranes was performed by dialysis as described (15) using dimyristoyl phosphatidylcholine (DMPC) as a lipid and 25 mM HEPES (pH 7.5) plus 3 mM NaN3 as a control. The overall conductance increase of the membrane. When dissolved in silver-stained 8% denaturing polyacrylamide gels. Matrix-assisted laser desorption ionization mass spectrometry confirmed that the purified MspA protein was composed of identical 20 kDa monomers, but the number of subunits or the molecular mass of the oligomer remained to be elucidated (9). To determine the stoichiometric composition of the functional MspA oligomer, we varied the purification procedure by using different detergents and organic solvents and applied mass spectroscopy using both matrix-assisted laser desorption ionization and electrospray ionization, but no signals with a mass-to-charge ratio larger than that of the MspA monomer were detected. In another approach, MspA was cross-linked via its accessible carboxyl groups using a water-soluble carbodiimide. MspA was significantly modified by the cross-linker as evidenced by the reduction of the electrophoretic mobility of MspA oligomers (Fig. 1, lane 4). This might be explained by the loss of negative surface charges because cross-linking between MspA oligomers is unlikely at a protein concentration of 150 nM (2.6 μg/ml), which is 20- to 1500-fold lower compared with other protocols used to obtain intermolecular cross-links (19). Denaturation of cross-linked MspA released four protein species (Fig. 1, lane 5) with apparent molecular masses of 21, 42, 64, and 83 kDa, i.e. multiples of the MspA monomer (20 kDa). Protein bands exceeding the apparent molecular mass of the native protein of 90 kDa were not observed neither in silver-stained gels nor by immunodetection of identical samples using an MspA antiserum (not shown). Molecular masses of 76 and 93 kDa were determined for oligomeric MspA by analytical gel filtration using both silica- and dextran-based column materials, respectively (not shown). The results of all these experiments were consistent with an MspA tetramer.

Pores in the Cell Wall of M. smegmatis—The structure of the M. smegmatis porins was investigated in their native environment, i.e. in isolated cell walls, by electron microscopy. In negatively stained preparations uranyl acetate-filled pores were clearly visible (Fig. 2A). The pore structures appeared...
randomly distributed at a density of $1010 \pm 310$ pores per $\mu m^2$ (determined from eight cell wall fragments containing 484 pores). Thus, the density of porins is about 15-fold less than that in the outer membrane of Gram-negative bacteria, in which more than 15,000 porin trimers per pores. The images were analyzed and rotationally aligned by principal component analysis, classified into three classes, averaged, and 4-fold symmetrized. Class I contains 398 images ($B$, class II 401 ($C$), and class III 268 images ($D$). Black areas represent the stain-filled pore, bright areas protein material. The resolution of class I and II averages is 1.7 nm according to the phase residual criterion. Size of averages: 14.5 nm.

Channel Structure of MspA—It is obvious that the porins of $M. \textit{smegmatis}$, and here MspA in particular, are radically different from trimeric porins of Gram-negative bacteria: MspA possesses only one channel per molecular complex (Fig. 2). Principal component analysis of 1757 individual pore images extracted from several micrographs of cell wall fragments revealed a 4-fold symmetric particle (Fig. 2, $B$ and $C$) that forms a central channel. Rotational alignment of the projections was performed with a bias-free approach using two related eigenvectors for orientational correction (18). The result is in perfect agreement with the tetrameric stoichiometry as deduced from the cross-linking and gel filtration experiments, and it excludes other compositions such as trimers or pentamers.

Classification yielded three sets of molecular images: particles with a pronounced 4-fold symmetric projection structure (Fig. 2, $B$ and $C$) and one class with poorly defined symmetry (Fig. 2D). The projections are consistent with a cylindrical molecule being partially embedded in stain and, by this way, enhancing the structure on one end or the other of the complex. The inner and outer diameter of class I molecules are 2.5 and 10 nm, respectively, and decreased to about 2.2 and 8 nm for class III molecules.

To obtain further structural information MspA was purified, reconstituted in the presence of DMPC (15), and analyzed by electron microscopy. Depending on the lipid-to-protein ratio a great variety of MspA supramolecular structures were observed: planar lipid membranes and lipid vesicles with randomly inserted MspA complexes, densely packed protein sheets, and spherical and, as depicted in Fig. 3A, linear MspA aggregates. Linear aggregates were obtained in top view and in side view projections (Fig. 3A). The latter show that the MspA pore complexes were arranged in two rows, intercalating like the teeth of a zipper. For image analysis, 20 individual MspA zippers were unbent in the computer, aligned by correlation methods, and averaged (Fig. 3, $B$ and $C$). The side views confirmed the cone-like structure of MspA as deduced from the top view averages and matched their dimensions, with an outer diameter of about 10 nm, one central open hole of 2.5 nm at one end converging to about 2.2 nm toward the other end (Fig. 4). The channel protein is $\sim$10 nm long. About 5.5 nm of the molecule were exposed to the polar environment and is well embedded in stain, thus, representing the more hydrophilic part of MspA. The remaining very hydrophobic portion of MspA is responsible for the assembly of the zipper-like structures.

MspA in Protein Micelles and Lipid Vesicles—To examine whether the apparent asymmetric surface hydrophobicity of the MspA complex is reflected in detergent-depleted solutions and in the lipid environment, octyl-POE was either removed or exchanged for DMPC by dialysis (15). Samples of purified and detergent-solubilized MspA that were extensively dialyzed against buffer or pure water did not yield insoluble precipitates. Instead they showed “protein micelles” in the electron
of Gram-negative bacteria form a single channel defined by one β-barrel per monomer. They occur as monomers such as the porin OmpG of E. coli (21) and the E. coli siderophore receptors FhuA (22) and FepA (23) or as trimers with three pores per molecule, e.g. OmpF of E. coli and related porins (24). Known tetrameric channel proteins belong to the α-type (25) and are located in the cytoplasmic membrane, e.g. the potassium channel (26). Another tetramer is formed by the black widow spider neurotoxin α-latrotoxin. However, this is a hydrophilic propeller-shaped 520-kDa channel, which only slowly inserts into lipid membranes in the absence of any receptor (27). Moreover, only a part of α-latrotoxin acquires amphiplicity upon oligomerization and is able to insert into membranes. By contrast, MspA is likely to be embedded over a considerable portion of its length of 10 nm in the mycobacterial outer membrane if the latter has indeed a thickness of 10 nm as proposed earlier (3, 28). We found evidence for a strongly hydrophobic portion of about 5 nm in length and assume that at least the outermost domain of ~2 nm thickness, which forms the 4-fold symmetry and is readily accessible to uranyl acetate, is exposed on the membrane surface in vivo. If the other part of MspA is lipid-embedded in vivo, MspA may possess a longer membrane-spanning domain than any other bacterial channel protein. Both the channel-tunnel TolC and the junctional pore complex of cyanobacteria are longer (14 and 32 nm, respectively), but their membrane-spanning domains do not exceed 4 nm (29, 30).

The Surface Hydrophobicity Gradient Is Responsible for the Oriented Interaction of the MspA Pore with Lipid Membranes—The formation of protein micelles and the MspA zippers illustrate the existence of a hydrophobicity gradient along the MspA pore with voluminous hydrophilic domains at one end and a strongly hydrophobic portion at the other. This appears to be another new characteristic of MspA because protein micelles have not yet been observed for other bacterial porins. The assumption that the hydrophobicity gradient of MspA would lead to an oriented interaction with hydrophobic surfaces was supported by the highly asymmetric insertion of MspA into planar lipid membranes. This property also explains the oriented adsorption of the MspA pore onto hydrophobic carbon surfaces (31).

The mycolic acids in the mycobacterial cell wall form tight crystalline arrays (32), which exhibit a drastically decreasing fluidity toward its periplasmic end (12). The asymmetrical surface hydrophobicity and membrane insertion might reflect the orientation of the MspA pore in the asymmetrical mycolic acid layer and might be important in vivo for gating. Porins of Mycobacterium chelonae and Mycobacterium phlei close asymmetrically at rather low negative voltages of ~40 and ~20 mV, respectively, in artificial membranes (33, 34) indicating that voltage gating might be a general property of porins from fast-growing mycobacteria. Because the existence of a Donnan potential across the mycobacterial cell wall similar to that observed in E. coli (35) was not experimentally demonstrated yet, it is not clear whether voltage gating of MspA in lipid bilayer experiments has any significance in vivo. However, the critical voltage of MspA, above which channels close, is only one third of the 90 mV, which is required to close the porin OmpF of E. coli (36). Moreover, there is growing evidence that a number of factors such as a low pH value (37) or the presence of polycations (38) or saccharides (39) reduce the critical voltage of porins from Gram-negative bacteria. Taking into account that charged lipids are only present in the outer layer of the mycolic acid membrane (40) it is tempting to speculate that a Donnan potential exists in mycobacteria and voltage gating of porins is exploited by mycobacteria to protect themselves from noxious molecules.
The Low Number of Porins and Their Structure Limit the Low Permeability of the Cell Wall of *M. smegmatis*—We have shown that the density of pore proteins in the cell wall of *M. smegmatis* is about 15-fold and that of single pores about 45-fold less compared with that of Gram-negative bacteria. However, this only partially accounts for the 50,000-fold lower permeability of the cell wall of *M. smegmatis* compared with *E. coli* for glucose (6). Molecular dynamics calculations of the diffusion of ions through the porin OmpF have shown that the diffusion constants are reduced to about 50% of their value in bulk solutions (41). Furthermore, diffusion rates through pore proteins decrease with the size of the substrate (42). Both effects are certainly more pronounced in the 2.5-fold longer MspA channel compared with the *E. coli* porins. Thus, the extraordinary length of the MspA pore hampers diffusion of hydrophilic compounds across the outer membrane of *M. smegmatis* further. A significant contribution might also originate from the putative periplasmatic end of the MspA channel, which was less filled with stain (Fig. 4) indicating either an extremely hydrophobic channel interior or a channel constriction. Both properties would exclude uranyl acetate from staining and would further reduce the pore permeability for polar molecules. In conclusion, the length, the cone-like structure of the MspA pore with its less permissive end and the low number of pores may explain why the outer membrane of *M. smegmatis* has such an extraordinary low permeability for hydrophilic compounds. Similar mechanisms likely restrict the porin pathway in *M. tuberculosis* because the thickness of the outer membrane determines the minimal length of the porins and appears to be similar for mycobacteria (28) and the number of porins appears to be low in *M. tuberculosis* (43).

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**Fig. 5.** Electron micrographs of reconstituted MspA. A, soluble MspA micelles obtained after removal of detergent by dialysis against water. B, image of a negatively stained DMPC vesicle containing reconstituted MspA. The protein complexes are concentrated at the vesicle border probably due to flattening of the vesicle on the specimen grid (inset). Side-on views of the MspA complex are visible at the vesicle border. Top views show the stain-filled (black) pore area of the molecules. Scale bars are 50 nm.

**Fig. 6.** Asymmetrical membrane insertion of purified MspA. 26 pg/ml of purified MspA was added to the cis-side of a diphytanoyl phosphatidylcholine membrane. Increasingly positive (upper traces) and negative voltages (lower traces) were applied to the membrane when ~100 channels were reconstituted. The membrane current was recorded.
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