Topography of the Porin MspA in the Outer Membrane of Mycobacterium smegmatis

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MspA is the major porin of Mycobacterium smegmatis mediating the exchange of hydrophilic solutes across the outer membrane (OM). It is the prototype of a new family of octameric porins with a single central channel of 9.6 nm in length and consists of two hydrophobic β-barrels of 3.7 nm in length and a more hydrophilic, globular rim domain. The length of the hydrophobic domain of MspA does not match the thicknesses of mycobacterial OM s of 5–12 nm as derived from electron micrographs. Further, the membrane topology of MspA is unknown as it is for any other mycobacterial OM protein. We used MspA as a molecular ruler to define the boundaries of the OM of M. smegmatis by surface labeling of single cysteine mutants. Seventeen mutants covered the surface of the rim domain and were biotinylated with a membrane-impermeable reagent. The label efficiencies in vitro were remarkably similar to the predicted accessibilities of the cysteines. By contrast, six of these mutants were protected from biotinylation in M. smegmatis cells. Tryptophan 21 defines a horizontal plane that dissects the surface-exposed versus the membrane-protected residues of MspA. The 8 phenylalanines at position 99 form a ring at the periplasmic end of the hydrophobic β-barrel domain. These results indicated that (i) the membrane boundaries of MspA are defined by aromatic girdles as in porins of Gram-negative bacteria and (ii) loops and a 3.4-nm long part of the hydrophilic rim domain are embedded into the OM of M. smegmatis. This is the first report suggesting that elements other than hydrophobic α-helices or β-sheets are integrated into a lipid membrane.

Mycobacteria are surrounded by an inner membrane and a giant macromolecule consisting of peptidoglycan, arabinogalactan, and very long chain fatty acids, the mycolic acids (1). Minnikin (2) originally proposed that the mycolic acids, which are covalently bound to the arabinogalactan-peptidoglycan co-polymer, form the inner leaflet of an unique outer membrane (OM).3 Experimental evidence for this model was provided by x-ray diffraction studies, which showed that the mycolic acids are oriented in parallel and perpendicular to the plane of the cell envelope (3). The presence of an organized structure of extremely low fluidity in an isolated mycobacterial cell wall was confirmed by differential scanning calorimetry (4). The vast abundance of non-covalently bound lipids in mycobacteria is assumed to constitute the outer leaflet of a supported asymmetric lipid bilayer. The analysis of spin-labeled fatty acids inserted into isolated mycobacterial cell walls by electron paramagnetic resonance supported the existence of a moderately fluid outer leaflet, whereas the inner leaflet had an extremely low fluidity (4, 5). This interpretation is consistent with observations that mutants with defects in the production of some of the major extractable lipids (glycopeptidolipids, phthiocerol dimycocerosates) showed an increased OM permeability (6, 7). Thus, the mycobacterial OM resembles a supported asymmetric lipid bilayer and provides an extraordinarily efficient permeability barrier, which is 100–1000-fold less permeable than that of Escherichia coli (8–10).

The existence of an additional lipid bilayer requires a set of dedicated OM proteins. E. coli uses more than 60 proteins to functionalize its OM (11), many of which are channel proteins to permeabilize the membrane for nutrient transport (12). The existence of pore-forming proteins in Mycobacterium chelonae was described 13 years ago (47), but substantial progress in terms of functional, biochemical, and structural properties of these porins has only been achieved for MspA, which was identified as a channel-forming protein in chloroform/methanol extracts of Mycobacterium smegmatis (30). Deletion of mspA reduced the OM permeability of M. smegmatis toward cephaloridine and glucose 9- and 4-fold, respectively (13). These results showed that MspA is the major general diffusion pathway for hydrophilic molecules in M. smegmatis. Electron micrographs of negatively stained cell wall fragments of M. smegmatis revealed MspA porins with a central channel of 10 nm in length and an tetra- or octameric symmetry (14). The core of the MspA channel is an extremely stable β-sheet domain, which resisted dissociation and denaturation up to 92 °C in the presence of 2% SDS as demonstrated by infrared spectroscopy (15). The first crystal structure of a mycobacterial OM protein showed that eight monomers of MspA constitute a single channel of 9.6 nm in length (16). This architecture is completely different from that of the trimeric porins of Gram-negative bacteria, which have one pore per monomer and are ~4 nm long (17). These results established MspA as the founding member of a new class of porins (18). Superficially, the length of MspA seems to fit the extraordinary thicknesses of the mycobacterial OMs, which were estimated to 5–12 nm from ultrathin sections of mycobacterial cells by electron microscopy (19–21). However, the hydrophobic part of MspA consists of two consecutive β-barrels of a combined length of only 3.7 nm (16). There are two alternative explanations: either the OM of M. smegmatis is not thicker than 3.7 nm, or the large and hydrophilic rim domain of MspA is at least partially embedded into the membrane.

To solve this puzzle, we examined, in this study, the topology of MspA in the OM of M. smegmatis by cysteine scanning mutagenesis and compared the accessibility of 47 cysteine mutants in vitro and in vivo with the membrane-impermeable biotinylation reagent N-(3-maleimidopropionyl)biotin (MPB). The results showed clear protection...
of cysteines in the hydrophilic rim domain in vivo, indicating that a larger part of MspA might be inserted into the OM of M. smegmatis than assumed from crystal structure.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Enzymes**—Chemicals were of the highest purity available from Merck, Roth, or Sigma. MPB was from Fluka. The detergent N-octylpolyoxyethylene (octyl-POE) was from Bachem. Oligonucleotides were obtained from MWG Biotech.

**Bacterial Strains and Growth Conditions**—M. smegmatis SMR5 (22) and M. smegmatis ML10, which lacks the porin genes mspA and mspC (23), were grown in 7H9 medium (BD Biosciences) supplemented with 0.2% glycerol and 0.05% Tween 80 at 37 °C. E. coli DH5α was used for cloning experiments and was routinely grown in Luria-Bertani broth (LB) at 37 °C. Hygromycin was used in concentrations of 0.5% (w/v) octyl-POE, boiled for 15 min, cooled on ice, and centrifuged for 10 min at 4 °C at 13,000 g. The supernatants were used directly or were stored at 20 °C.

**Site-directed Mutagenesis**—Cysteine mutants of MspA were constructed by site-specific mutagenesis using the combined chain reaction method (24), the plasmid pMN016, which carries the pms gene—mspA transcriptional fusion (23) as template, and psmc1 and pMS-seq1 as end primers and an appropriate mutagenesis primer (supplemental Table S1). All plasmids were verified by sequencing the entire mspA gene before they were transformed into the porin mutant M. smegmatis ML10.

**Analysis of the Total Protein of M. smegmatis**—M. smegmatis containing the mspA expression plasmids or the empty vector pMS2 (25) as a control was grown to an optical density (A600) of 0.8. Cells were harvested by centrifugation at 3000 × g for 10 min at 4 °C and were washed three times with PEN buffer (100 mM NaH2PO4/Na2HPO4, pH 6.5, 0.1 mM Na2EDTA, 150 mM NaCl). Then, the cells were suspended in 1 ml of PEN buffer to yield an optical density A600 of 8. 500 μl of the cell suspension were mixed with suspended glass beads (volume ratio 3:1). The cells were disrupted with a bead-beater (FastPrep FP120, Qbiogene Inc., intensity 6, 2 × 20 s). The suspension was adjusted to a final concentration of 0.5% (w/v) octyl-POE, boiled for 15 min, cooled on ice, and centrifuged for 10 min at 4 °C at 13,000 × g. Protein concentrations in the supernatant were determined by the bicinchoninic acid (BCA) assay according to the manufacturer’s instructions (BCA protein assay kit, Pierce). The supernatants were used directly or were stored at −20 °C. For analysis, the proteins were separated on denaturing 10% polyacrylamide gels followed by staining with Coomassie Brilliant Blue.

**Expression and Selective Extraction of Porins from M. smegmatis**—The wild-type (WT) mspa gene (pMN016) and the mutated mspa genes (pMN016 derivatives) were constitutively expressed in the M. smegmatis porin mutant ML10. All MspA proteins were extracted from M. smegmatis using a method that is selective for Msp porins (26). Briefly, cells were harvested and washed three times with 5 ml of ice-cold phosphate-buffered saline (PBS) (100 mM NaH2PO4/Na2HPO4, 0.1 mM Na2EDTA, 150 mM NaCl, 0.05% (w/v) Tween 80, pH 7), resuspended in POP05 buffer (300 mM NaH2PO4/Na2HPO4, 0.3 mM Na2EDTA, 150 mM NaCl, 0.5% (w/v) N-octyl-POE) to a cell density of 10 mg/ml cells (wet weight), and boiled under stirring for 30 min in a water bath. The suspension was cooled on ice for 10 min and centrifuged at 4 °C for 10 min at 13,000 × g. In wild-type M. smegmatis, Msp porins constitute more than 80% of the total protein content in the supernatant (26). The proteins were separated in a denaturing 10% polyacrylamide gel, which was stained with silver.

**Optimization of the Biotinylation Reaction**—To optimize the biotinylation reaction, a 20-ml culture of M. smegmatis expressing the MspA cysteine mutant E139C was grown to an A600 of 0.8. The cells were harvested at 4000 rpm at 4 °C for 10 min and washed twice with PBS. The pellet was resuspended in 2 ml of PBS, and dithiothreitol (DTT) was added to a final concentration of 10 mM. The suspension was incubated for 30 min at room temperature followed by centrifugation at 4000 rpm at 4 °C for 10 min. The pellet was washed with PBS and resuspended in 2 ml of PBS. Samples of 200 μl were incubated for 30 min at 25 °C at final MPB concentrations of 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 mM. The reaction was stopped by adding DTT to a final concentration of 20 mM. A concentration of 0.1 mM was sufficient to clearly detect the biotinylated MspA mutant in Western blot experiments. Then, the minimal reaction time was determined by carrying out the biotinylation in the presence of 0.1 mM MPB. At different times, 200-μl samples were taken from the mixture, and the reaction was stopped at the indicated times with DTT as described above. All samples were analyzed by Western blot experiments.

**Biotinylation of Denatured MspA Cysteine Mutants**—To verify the presence of a cysteine in the MspA mutants, MspA in selective extracts of M. smegmatis was denatured by adding ethanol to a final concentration of 80% (v/v) and heating at 100 °C for 15 min. Ethanol was removed by vacuum, and the pellet was dissolved in POP05 buffer (same volume as the original extract volume). The sample was then incubated with DTT to reduce oxidized cysteines (see above). To remove the DTT, the protein was precipitated after adding an equal volume of acetone and incubation on ice for 15 min. After centrifugation for 10 min at 4 °C at 13,000 × g, the pellet was dissolved in 10 μl of POP05. The labeling reaction was carried out by adding MPB to a final concentration of 0.1 mM and incubating for 15 s at 25 °C. The reaction was stopped by adding DTT to a final concentration of 20 mM.

**Biotinylation of Native MspA Cysteine Mutants in Vitro and in Vivo**—For biotinylation of the MspA cysteine mutants in vitro and in vivo cells were grown to an optical density A600 of 0.8, harvested by centrifugation, and washed three times with 2 ml of ice-cold PBS (100 mM NaH2PO4/Na2HPO4, 0.1 mM Na2EDTA, 150 mM NaCl, 0.05% (v/v) Tween 80, pH 7). To compare the label efficiencies for the MspA cysteine mutants in vivo and in vitro, a culture of M. smegmatis expressing a particular MspA mutant was split into two halves. The respective MspA mutant protein was extracted at 100 °C from one half of the culture. To reduce potentially oxidized cysteines, 10 μl of the extracts were incubated with DTT at a final concentration of 10 mM for 30 min at 25 °C. Removal of DTT by precipitation of the proteins with acetone and biotinylation with MPB was done as described in the previous paragraph. For in vivo biotinylation, the other half of the cells was incubated with DTT at a final concentration of 10 mM for 30 min at 25 °C. The cells were washed again with PBS, and 200 μl of the cell suspension were incubated at 25 °C in a final concentration of 0.1 mM MPB. The reaction was stopped after 15 s by adding DTT to a final concentration of 20 mM. After washing with PBS and resuspending in POP05 buffer (300 mM NaH2PO4/Na2HPO4, 0.3 mM Na2EDTA, 150 mM NaCl, 0.5% (v/v) N-octyl-POE) to a cell density of 10 mg/100 μl, the suspension was boiled under stirring for 30 min in a water bath. The suspension was cooled on ice for 10 min and centrifuged at 4 °C for 10 min at 13,000 × g, and the pellet containing the cell debris was discarded. The supernatant contained the MspA mutant protein. Both supernatants, obtained after biotinylation of the MspA mutant protein in vitro or within M. smegmatis cells, were further analyzed for protein content by gel electrophoresis and for the amount of biotin label by Western blot experiments.

**Gel Electrophoresis and Western Blots**—Protein samples were analyzed in denaturing polyacrylamide gels, which were stained with Coomassie Blue or silver. All samples were mixed with loading buffer (140 mM Tris, 4% SDS, 30% glycerol, 0.1% bromphenol blue, pH 7.5) and incubated for 10 min at room temperature before loading on a denatur-
**Topology of Porin MspA in Outer Membrane of M. smegmatis**

![Schematic representation of the secondary structure of MspA and the residues mutated to cysteine.](image)

**RESULTS**

Construction of MspA Cysteine Mutants—The versatility of single cysteine mutants appeared to be most attractive to investigate the membrane topology of the MspA porin. Since wild-type MspA does not contain any cysteine residues, 52 out of 184 amino acids of the mature protein were directly replaced by cysteines using site-directed mutagenesis (Fig. 1). The positions for these mutations were selected before the crystal structure of MspA was published (16) using the following criteria: (i) Putative extracellular loops were predicted by a neural network specialized for outer membrane proteins (28) and by an algorithm specialized for prediction of membrane-spanning β-strands (29, 30) and were preferably targeted to gain topological information. (ii) Charged amino acids are often located in extracellular loops and may also play functional roles by determining the charge selectivity of MspA. Thus, 20 out of 28 charged amino acids were mutated. (iii) The four known Msp porins of *M. smegmatis* constitute a family of very closely related proteins, which differ altogether at 18 positions from a consensus sequence (13). These positions might be functionally important and were mutated except for the conservative exchanges L8V, I68V, S103T, and V104I. (iv) The remaining positions were selected to cover the entire MspA sequence without gaps larger than eight amino acids.

Expression of MspA Cysteine Mutants in *M. smegmatis*—The mutated *mspA* genes were expressed in the *M. smegmatis* strain ML10, which lacks the two porin genes *mspA* and *mspC*. This strain contains a 15-fold reduced number of Msp porins in the OM (23). To examine whether the MspA cysteine mutants were expressed in the OM of *M. smegmatis*, a selective extraction procedure was employed that yields predominantly MspA when whole cells of *M. smegmatis* are heated with 0.5% octyl-POE to 100 °C (26). The amount of all 52 MspA cysteine mutants in these selective heat extracts were examined by gel electrophoresis. An example is shown in Fig. 2. It was shown earlier that expression of WT MspA in the ML10 strain using the vector pMN016 (p*amyC*-mspA transcriptional fusion) yields MspA levels as in WT *M. smegmatis* (23). No MspA or other Msp protein was observed in extracts of the ML10 strain with an empty vector as a control in Coomasie Brilliant Blue-stained gels (Fig. 2, lane 3), indicating a very low background expression of other *msp* genes. A surprisingly high number of 36 mutants showed a significantly reduced or no detectable expression when compared with WT MspA (Fig. 2, lane 2). Examples are the mutants A96C and H148C shown in Fig. 2 (lanes 4 and 8). Only 16 out of 52 cysteine mutants were expressed on a similar level as WT MspA (not shown). Examples are five mutants shown in Fig. 2 (D134C, E139C, G141C, T150C, and A155C). The MspA octamer was observed in Coomasie Brilliant Blue-stained polyacrylamide gels for all cysteine mutants, albeit often as barely detectable bands, e.g. as for the D3C mutant (supplemental Fig. S1). However, 13 mutants were clearly less stable than the WT protein as evidenced by an increased amount of MspA monomer (supplemental Fig. S1 and Table 1). It should be noted that even for those destabilized mutants, the major part of the total protein was oligomeric after extraction for 30 min at 100 °C.

**Detection of Cysteines by Biotinylation of Denatured MspA Mutant Proteins**—To verify the presence of a cysteine, all MspA mutant proteins in selective extracts of *M. smegmatis* were denatured by heating to...
Topology of Porin MspA in Outer Membrane of M. smegmatis

### TABLE 1

Characterization of cysteine mutants of MspA

| Mutation | Location | % Accessibility | Biotinylation | Stability |
|----------|----------|-----------------|---------------|-----------|
|          |          |                 | Denatured     | In vitro  |
| WT       |          |                 | +             | O         |
| G1C      | Rim      | 45              | +             | O         |
| D5C      | Rim      | 27              | +             | O         |
| E5C      | Rim      | 24              | +             | O         |
| D10C     | Rim      | 2               | +             | O         |
| D13C     | Rim      | 32              | +             | ND        |
| R14C     | Rim      | 5               | +             | M         |
| T17C     | Rim      | 9               | +             | O         |
| W21C     | Rim      | 7               | +             | O         |
| D22C     | Rim      | 24              | +             | O         |
| G27C     | Stem     | 24              | +             | ND        |
| R33C     | Rim      | 35              | +             | M         |
| R38C     | Rim      | 6               | +             | M         |
| G44C     | Rim      | 1               | +             | O         |
| K47C     | Rim      | 12              | +             | O         |
| I49C     | Rim      | 5               | +             | O         |
| E139C    | Rim      | 38              | +             | M         |
| D134C    | Rim      | 47              | +             | O         |
| A138C    | Rim      | 12              | +             | O         |
| E139C    | Rim      | 38              | +             | O         |
| G141C    | Rim      | 10              | +             | O         |
| V141C    | Rim      | 3               | +             | O         |
| H148C    | Rim      | 22              | +             | M         |
| T150C    | Rim      | 21              | +             | M         |
| A155C    | Rim      | 12              | +             | M         |
| R16C     | Rim      | 0               | +             | M         |
| R165C    | Rim      | 3               | +             | M         |
| S173C    | Rim      | 9               | +             | M         |
| T175C    | Rim      | 1               | +             | M         |
| E179C    | Rim      | 44              | +             | O         |
| N184C    | Rim      | 40              | +             | O         |

Biotinylation of denatured and oligomeric MspA was analysed *in vitro* using MPB. The relative accessibility of the cysteine residues towards solvents in the x-ray structure was analyzed by the accessibility function of Swiss-pdbViewer. The status of MspA after biotinylation *in vitro* is indicated by M or O (bold), if the major part of the protein was observed by gel electrophoresis as monomer or oligomer, respectively. A reduced thermostability of a mutant was indicated by an increased amount of MspA monomer in Coomassie Blue-stained Protein gels (Fig. S1). This was observed for 12 mutants, which are marked with M in the column "stability." ND., not determined.

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100 °C in the presence of 80% ethanol. Reaction with the thiol-specific MPB and detection of the biotinylated MspA proteins in Western blot experiments using streptavidin demonstrated that all but two MspA cysteine mutants (G27C, T150C) were indeed expressed in *M. smegmatis*, albeit some at levels undetectable in protein gels by Coomassie Blue and silver staining (not shown). It is not clear why the G27C and T150C mutants were not biotinylated after denaturation because both proteins were clearly detectable in detergent extracts (supplemental Fig. S1). Wild-type MspA protein was not biotinylated because it does not contain a cysteine and was used as a negative control in those experiments. No or very faint bands of biotinylated proteins other than the respective MspA cysteine mutants were observed in these experiments, indicating that cysteine-containing proteins were almost completely absent in the MspA-selective detergent extracts of *M. smegmatis*.

**Optimization of the Biotinylation Conditions**—It was crucial to ensure that the biotinylation reaction was rate-limited to quantify the accessibility of the cysteines in the MspA mutants. The E139C mutant was chosen for these experiments because this residue is surface-exposed as demonstrated using a whole cell enzyme-linked immunosorbent assay experiment (16). The rationale was that the rate-limiting conditions for this residue should also be rate-limiting for other, less accessible cysteines. To this end, the amount of the label reagent MPB and the reaction time should be reduced as much as possible. The MspA mutant E139C was incubated with MPB *in vitro* at final concentrations ranging from 0.05 to 10 mM for 30 min. Biotinylated MspA was clearly detected in samples containing 50 μM and higher concentrations of MPB in Western blot experiments (supplemental Fig. S2A). A kinetic experiment using 100 μM MPB revealed that incubation for 15 s was sufficient to detect biotinylation of the cysteine at position 139 (supplemental Fig. S2B). Thus, in all following experiments, the MspA cysteine mutants were labeled *in vitro* or in whole cells of *M. smegmatis* with 100 μM MPB for 15 s at 25 °C unless otherwise noted.

**Biotinylation Efficiencies Reflect Altered Accessibilities to MPB of Cysteines of Membrane-embedded MspA**—To determine which cysteines are accessible to the membrane-impermeable biotinylation reagent MPB in the native OM environment of MspA, the MspA mutants were expressed in *M. smegmatis* ML10 (ΔmspA ΔmspC). To ensure that equal amounts of MspA protein were present in both the *in vitro* and the *in vivo* biotinylation reactions, a culture of *M. smegmatis* expressing a particular MspA mutant was split into two halves. One half of the cells was labeled with MPB before the MspA protein was selectively extracted from the cells (*in vivo* samples). MspA protein was extracted from the other half of the cells and was then labeled with MPB (*in vitro* samples). Labeling and extraction of the MspA mutants in both series of samples were done using identical conditions. The amount of MspA protein in the *in vitro* and *in vivo* samples was indistinguishable in Coomassie Brilliant Blue-stained polyacrylamide gels for all cysteine mutants except for the R38C mutant (supplemental Fig. S1). This test was used to control the homogeneity of the samples collected for comparison in the *in vitro* and *in vivo* experiments. Thirty-nine out of fifty-two cysteine mutants were labeled *in vitro*.
vitro with 100 μM MPB for 15 s (supplemental Fig. S4 and Table 1). Since the MspA octamer is the channel-forming unit in the OM of M. smegmatis (14), a meaningful comparison of the accessibility of cysteines in vitro and in vivo is only possible for mutants, which are predominantly labeled as oligomers. Therefore, all mutants, which were only or mainly (A96C, G100C) labeled as monomers, were excluded from further analysis. Obviously, labeling of cysteines that are buried inside the protein, in particular inside the large rim domain, cannot occur. This was the case for 10 mutants (G44C, G60C, E63C, G69C, R38C, V144C, R161C, R165C, S173C, T175C), which had a predicted accessibility of less than 10% and showed no label. This result may be explained by strong association of membrane lipids with the hydrophobic β-barrels of MspA after protein extraction as observed earlier (15). Thus, we focused on 18 mutants, the oligomer of which was the only or the major band after biotinylation in vitro (Fig. 3). Quantitative image analysis showed that the in vitro label efficiencies of cysteines of these mutants are remarkably similar to the accessibility values of the respective thiol groups as predicted from the crystal structure (supplemental Fig. S3). These results indicated that (i) cysteine replacement did not cause a major distortion of the structure of these MspA mutants and (ii) labeling with MPB was indeed a good measure for the accessibility of these particular residues within the MspA protein under those conditions.

The cysteines of the vast majority of these selected MspA mutants were clearly less efficiently labeled in vitro than in vitro (Fig. 3). Slightly or strongly enhanced label efficiencies in vivo were only observed for the mutants W21C and K47C, indicating that these cysteines are highly accessible in the OM of M. smegmatis. These mutants showed further the validity of the use of MPB for probing the accessibility of cysteines in MspA in M. smegmatis. Since the amount of protein in the in vivo and in vitro samples was identical for all mutants tested (Fig. 3), it is concluded that the different biotinylation efficiencies by MPB in vivo reflect the altered accessibilities to the respective cysteines of membrane-embedded MspA.

Tryptophan 21 Defines a Horizontal Plane That Dissects Protected Cysteines from Accessible Cysteines—Antibodies generated against the MspA peptide (127EVATFSVDDGAEG146) recognized whole cells of M. smegmatis in enzyme-linked immunosorbent assay experiments and defined this epitope as surface-accessible (16). However, in vivo biotinylation of the four mutants D134C, S136C, A138C, and E139C, which are located in this epitope, was considerably reduced (Fig. 3). Quantitative image analysis of the blots shown in Fig. 3 yielded protection values between 36 and 61% for these mutants (Fig. 4). This indicates that surface structures of M. smegmatis may reduce the accessibility of these residues to MPB. Consequently, a cut-off value of 65% relative protection in vivo was assigned to accessible cysteines. Five other mutants (G1C, T17C, I49C, E57C, and G141C) fall into the same accessibility class II. Taken together with the two highly accessible cysteines at positions 21 and 47, 11 out of 18 MspA mutants were accessible to biotinylation by MPB in vivo (classes I and II). These cysteines are exclusively located in the upper half of the rim domain of MspA (Figs. 5 and 6). Surprisingly, we observed complete or almost complete protection for the mutants D3C, E5C, D22C, E179C, and N184C (class III) within the hydrophilic rim domain (Figs. 3–5). Most remarkably, tryptophan 21 defines a horizontal plane that dissects the 5 protected cysteines from the accessible cysteines (Fig. 5). This indicates that the residues below tryptophan 21 are embedded into the membrane and are shielded from MPB by OM lipids. Furthermore, the cysteine at position 5 is very close to the presumed borderline defined by Trp21 and shows a slight, but significant, biotinylation in contrast to the cysteines at positions 3, 22, 179, and 184. This is consistent with the expectation that cysteines, which are more deeply buried in the membrane, should be less accessible to modification by the hydrophilic MPB. Only two mutants apparently do not fit to this theory. Cysteine 91 is completely protected in vivo. However, residue 91 forms the lower ring of the constriction zone, which is located at the periplasmic end of MspA. Labeling of the cysteine 96 in the periplasmic loop in vivo demonstrated that MPB can diffuse through the constriction zone; however, the signal is weak when compared with that of the A96C mutant labeled in vitro, indicating that diffusion of MPB through MspA is rate-limiting in this case (supplemental Fig. S4). The same argument likely applies to the D91C mutant.

![Figure 3](https://example.com/fig3.png)
Topography of Porin MspA in Outer Membrane of M. smegmatis

DISCUSSION

The MspA structure is unlike that of any other known protein, and its membrane topology is unknown as it is for any other mycobacterial OM protein. In this study, we used MspA as a molecular ruler to define the boundaries of the OM of M. smegmatis by surface labeling of single cysteine mutants. The thermal stability of 13 and the expression level of mutation values above 65% were categorized as inaccessible. All mutants with protection values above 65% were categorized as inaccessible. Class I, Trp21 (accessible: ~100 to 20%), Class II, G1C, T17C, I49C, E57C, D134C, S136C, A138C, E139C, G141C (partially accessible: 20–65%). Class III, D3C, E5C, D10C, D22C, D91C, E179C, N184C (protected: 80–100%). The atomic coordinates of M. smegmatis were taken from the crystal structure (PDB accession code: 1UUN) (16). The amino acids, which were mutated to cysteines and were labeled as oligomers in vitro (Fig. 3), are shown in ball-and-stick representation and are marked with their single-letter code. Carbon, oxygen, and nitrogen atoms are shown in gray, red, and blue, respectively. The amino acids, Asp10, Thr17, Asp134, Ser136, and Gly141 are not visible because they are covered by surface residues. The long loop at the bottom end of MspA has been named L6 (Fig. 1) and likely faces the periplasm of M. smegmatis. The figure was generated using ViewerLite (Accelrys, San Diego, CA).

The cysteine at position 10 is the only inaccessible residue that is located above Trp21. Since Asp10 is not a surface residue, its protection cannot be explained by membrane insertion directly. However, Asp10 is deeply buried in a pocket formed by loops L1 and L3 of the same monomer and the strands β9 and β10 of the neighboring monomer. Conformational changes of the surface loops may lead to the protection of this residue in vivo.

The Hydrophobic β-Barrels of MspA Are Very Likely Embedded into the Outer Membrane of M. smegmatis—Strikingly, 17 out of 18 cysteines, which were labeled in oligomeric proteins in vitro, are located in the hydrophilic rim domain, whereas none of the 10 cysteines in the hydrophobic stem domain were labeled. Since almost all of these residues were predicted as accessible in the crystal structure, these results suggested that the hydrophobic β-barrel domain of MspA is covered by other molecules and therefore protected from labeling. This assumption is supported by the observation using infrared spectroscopy that MspA is associated with a 10-fold molar excess of lipids even after extensive purification. These lipids did not dissociate from MspA in the presence of 2% SDS and were only partially removed after precipitation with acetone, indicating that they are strongly bound by MspA. This is consistent with the finding that the hydrophobic stem domain of MspA is embedded into dimyristoyl phosphatidylcholine membranes in vesicles (14). These results leave little doubt that the β-barrels of MspA are also inserted into the OM of M. smegmatis as expected from its very hydrophobic surface (16), although we have no direct experimental evidence for this conclusion.

The Rim Domain of MspA Is Partially Membrane-embedded—The 17 in vitro accessible cysteines cover the entire surface of the rim domain of MspA (Fig. 5 and 6). Surprisingly, 6 cysteines were protected in vivo using the membrane-impermeable reagent MPB, whereas 11 cysteines were labeled (Fig. 3 and 5). Among the labeled cysteines were those at positions 134–141, which were characterized as surface-accessible by enzyme-linked immunosorbent assay experiments with whole cells of M. smegmatis using a peptide-specific antibody (16). These results clearly defined an area of the rim domain that was accessible to surface labeling by MPB and another area that was protected from MPB in vivo. These areas were separated by a plane through tryptophan 21. The ring of the 8 tryptophans at position 21 is reminiscent of the girdles of aromatic amino acids, which border the non-polar surfaces of porins of Gram-negative bacteria (33, 34). The observation that the cysteines at the adjacent positions 21 and 47 are the only residues with an enhanced label efficiency in vivo (Fig. 3 and 5) may be explained by a higher local concentration of the MPB reagent due to adsorption to the surface of

4 H. Engelhardt and M. Niederweis, unpublished data.
M. smegmatis and is, therefore, consistent with the assumption that the tryptophans 21 of MspA are located at the lipid-water interface.

Aromatic Girdles Appear to Determine the Topology of MspA in the Outer Membrane of M. smegmatis—The membrane-spanning domain of porins of Gram-negative bacteria consists of a central belt of non-polar residues bordered by girdles of aromatic amino acids with a vertical separation of 2–2.5 nm (33, 34). It has been suggested that these aromatic residues are especially adapted to accommodate the abrupt change of the dielectric properties at the acyl-water interface (33), to shield the porin against slow membrane movements (34), and to mechanically stabilize the protein (35). Tryptophans are also postulated to prevent aggregation of porins inside the OM (36). Molecular dynamics simulations showed that the aromatic rings of the tryptophan and tyrosine residues on the outside of OmpF are oriented predominantly perpendicular to the bilayer plane. The phenylalanines of OmpF are more mobile and assume a variety of orientations (37). The tryptophan 21 of MspA is also perpendicular to the bilayer plane, indicating that it may provide similar functions to MspA as discussed for the aromatic girdles of OmpF above. The same holds true for phenylalanine 99, which is located at the end of the hydrophobic β-barrel domain of MspA and likely lies at the interface of the OM and the periplasm. Thus, it appears that aromatic girdles define the topology of MspA in the OM of M. smegmatis.

Several further aromatic surface residues were identified in MspA (Fig. 6), which are oriented perpendicular to the bilayer plane and may therefore in principle provide the functions of aromatic girdles as discussed above. The most unusual aromatic girdle consists of a “tetrad” formed by Tyr177 of one monomer and by Phe41, Phe41, and Trp181 of the adjacent monomer (supplemental Fig. S5). Each of these residues are located in different β-strands. This aromatic girdle defines a plane, which is 1.4 nm below the presumed OM boundary. The outer leaflet of mycobacterial OM is known to contain short chain lipids (C16–C18) such as the diacyl and triacyl trehaloses (38) as well as longer lipids such as trehalose dimycolates (38–40). Since short chain lipids are about 1.2–1.5 nm long, we propose that this particular aromatic girdle additionally anchors MspA between the inner and outer leaflet of the OM of M. smegmatis. This area is characterized by an abrupt change from the outer leaflet with a high lipid fluidity to the inner layer of covalently bound mycolic acids with an extremely low lipid fluidity as observed by spin-labeled fatty acids in isolated cell walls of M. chelonae (4).

How Are Loops and Charged Amino Acids of MspA Integrated into a Membrane?—So far, the only known transmembrane structural motifs are hydrophobic α-helices for inner membrane proteins and amphiphilic β-sheets for outer membrane proteins (17). It has been concluded from these findings that a transmembrane polypeptide segment can traverse the membrane only if it has a hydrophobic outside and a complete backbone-backbone hydrogen bonding as in α-helices and β-sheets (41, 42). Superficially, MspA follows this pattern because it possesses two amphiphilic β-barrels with a hydrophobic outside and a hydrophilic interior (16). However, the crystal structure shows that the barrel-forming β-strands are connected by the loops L5, L5’, and L7. Furthermore, our results strongly indicate that in addition, the loops L2, L4, L8, L10, L11, and L12 (Fig. 1) are embedded into the OM of M. smegmatis. The loops L4, L5, L8, L10, and L11 are composed of two or three mostly hydrophobic residues. By contrast, 3 out of 9 residues of the L2 loop and 1 out of 3 residues of the L12 loop are charged (Fig. 1). Charged amino acids within β-strands of the rim domain such as Asp3, Glu3, Asp10, Asp22, and Glu179 are highly protected from modification by an membrane-impermeable reagent in M. smegmatis (Fig. 4 and 6) and would also be exposed to membrane lipids according to the model presented in Fig. 6. It is unknown and under further investigation how MspA accommodates charged residues and loops with apparently accessible hydrogen bond donors and acceptors within the OM of M. smegmatis. However, several mechanisms may account for this phenomenon. (i) The charged residues and loops may be involved in salt bridges and hydrogen bonding, respectively, with other molecules within the membrane. For example, the glutamate 32 is part of a trans-

**FIGURE 6.** Membrane topology of MspA as defined by surface labeling of single cysteines of MspA. The left side of the figure shows a surface model of the MspA porin (PDB accession code: 1UUN) to visualize the protection of surface cysteines to biotinylation in vivo according to the analysis shown in Fig. 4. Residues in dark and light green have protection values less than 30 and 65%, respectively, when compared with biotinylation in vitro and are defined as accessible. Residues in red have protection values less than 80% and are considered as inaccessible. The right side of the figure shows surface-exposed aromatic residues of MspA. Tryptophans are colored red, tyrosines are colored green, and phenylalanines are colored blue. Trp72, Phe110, and Phe29 are omitted from the surface model for clarification. Both figures were generated using PYMOL (DeLano Scientific, South San Francisco, CA).
membrane helix of the chicken ubiquinol cytochrome c and may interact with a lipid head group (43). (ii) The energetically very unfavorable exposition of charged amino acids to the aliphatic side chain of membrane lipids (41, 44) may drive a conformational change of the flexible loops and thereby induce interactions with the protein backbone. (iii) Large pK_a shifts have been observed in several instances both for aspartates and for arginines within proteins or in model peptides when compared with the free amino acids (45, 46). Thus, some of the arginines, aspartates, and glutamates in membrane facing loops of MspA may not be charged in their native environment.

Taken together, the results of this study strongly indicate that a larger part of MspA is inserted into the OM of M. smegmatis than inferred from the surface hydrophobicity (16). Clearly, more direct experimental approaches are needed to support this conclusion.

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REFERENCES
1. Brennan, P. J., and Nikaido, H. (1995) Annu. Rev. Biochem. 64, 29–63
2. Minnikin, D. E. (1982) in The Biology of the Mycobacteria: Physiology, Identification and Classification (Ratledge, C., and Stanford, J., eds) pp. 95–184, Academic Press, London.
3. Nikaido, H., Kim, S. H., and Rosenberg, E. Y. (1991) Mol. Microbiol. 5, 1125–1128
4. Li, J., Barry, C. E., III, Besra, G. S., and Nikaido, H. (1996) J. Bacteriol. 178, 3017–3023
5. Liu, J., Rosenberg, E. Y., and Nikaido, H. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 11254–11258
6. Liu, J., Barr, C. E., III, Besra, G. S., and Nikaido, H. (1996) J. Biol. Chem. 271, 29545–29551
7. Chambers, H. F., Moreau, D., Yajko, D., Muck, C., Wagner, C., Hackbart, C., Kocagöz, S., Rosenberg, E., Hadley, W. K., and Nikaido, H. (1995) Antimicrob. Agents Chemother. 39, 2620–2624
8. Molloy, M. P., Herbert, B. R., Slade, M. B., Rabilioud, T., Nouwens, A. S., Williams, K. L., and Gooley, A. A. (2000) Eur. J. Biochem. 267, 2871–2881
9. Nikaido, H. (2003) Microbiol. Mol. Biol. Rev. 67, 593–656
10. Stahl, C., Kubetzko, S., Kaps, I., Seeber, S., Engelhardt, H., and Niederweis, M. (2001) Mol. Microbiol. 40, 451–464; Correction (2005) Mol. Microbiol. 57, 1509
11. Engelhardt, H., Heinz, C., and Niederweis, M. (2002) J. Biol. Chem. 277, 37567–37572
12. Heinz, C., Engelhardt, H., and Niederweis, M. (2003) J. Biol. Chem. 278, 8678–8685
13. Faller, M., Niederweis, M., and Schulz, G. E. (2004) Science 303, 1189–1192
14. Koebnik, R., Locher, K. P., and van Gelder, P. (2000) Mol. Microbiol. 37, 239–253
15. Niederweis, M. (2003) Mol. Microbiol. 49, 1167–1177
16. Paul, T. R., and Beveridge, T. J. (1992) J. Bacteriol. 174, 6508–6517
17. Paul, T. R., and Beveridge, T. J. (1994) Infect. Immun. 62, 1542–1550
18. Sander, P., Prammananan, T., and Böttger, E. C. (1996) Mol. Microbiol. 22, 841–848
19. Stephan, J., Bender, J., Wolschendorf, F., Hoffmann, C., Roth, E., Mairland, C., Engelhardt, H., and Niederweis, M. (2005) Mol. Microbiol. 58, 714–730
20. Li, W., and Starnbrook, P. J. (1998) Annu. Rev. Biochem. 67, 137–140
21. Kaps, I., Ehr, S., Seeber, S., Schnappinger, D., Martin, C., Riley, L. W., and Niederweis, M. (2005) Gene (Amst.) 375, 115–124
22. Heinz, C., and Niederweis, M. (2000). Annu. Rev. Biochem. 69, 313–330
23. Schägger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
24. Diederich, K., Freigang, J., Umhau, S., Zeth, K., and Breed, J. (1998) Protein Sci. 7, 2413–2420
25. Rauch, G., and Moran, O. (1994) Biochem. Biophys. Res. Commun. 200, 908–915
26. Schulz, G. E. (1993) Curr. Opin. Cell Biol. 5, 701–707
27. Kreusch, A., and Schulze, G. E. (1994) J. Mol. Biol. 243, 891–905
28. Killian, J. A., Salemink, I., de Plancq, M. R., Lindblom, G., Koepp, R. E., II, and Greathouse, D. V. (1996) Biochemistry 35, 1037–1045
29. Tieleman, D. P., and Berendes, H. J. (1998) Biophys. J. 74, 2786–2801
30. Minnikin, D. E., Kremer, L., Dover, L. G., and Besra, G. S. (2002) Chem. Biol. 9, 545–553
31. Russell, D. G., Mwandumba, H. C., and Rhomeas, E. E. (2002) J. Cell Biol. 158, 421–426
32. Chatterjee, D., and Kho, K. H. (2001) CMLS Cell. Mol. Life Sci. 58, 2018–2024
33. White, S. H., and Wimsley, W. C. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 319–365
34. White, S. H., Ladozhkin, A. S., Jaysinghe, S., and Hristova, K. (2001) J. Biol. Chem. 276, 32395–32398
35. Ullrichs, S. M., and Saxon, M. S. (2001) Biochim. Biophys. Acta 1512, 1–14
36. Wimsley, W. C., Creamer, T. P., and White, S. H. (1996) Biochemistry 35, 5109–5124
37. Urry, D. W., Gowda, D. C., Peng, S., Parker, T. M., Jing, N., and Harris, R. D. (1994) Biopolymers 34, 889–896
38. Zachariae, U., Koshimoto, A., Engelhardt, H., and Karshikoff, A. (2002) Protein Sci. 11, 1309–1319
39. Trias, J., Jarlier, V., and Benz, R. (1992) Science 258, 1479–1481