

Characterization of a Porin from *Mycobacterium smegmatis*

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A pore-forming protein with an M_r of 40,000 has been extracted from the cell wall of *Mycobacterium smegmatis* with buffer containing the detergent Zwittergent 3-12 and 0.5 M NaCl and purified on an anion-exchange column. Although the pore diameter was large (2 nm), the specific activity was much lower than those of nonspecific porin channels of enteric bacteria. The channel allowed the permeation of small hydrophilic molecules such as sugars and amino acids. Its N-terminal sequence did not show any similarity to those of other porins sequenced so far.

Members of the genus *Mycobacterium* cause diseases such as tuberculosis and leprosy and are intrinsically resistant to a wide range of antibiotics (2, 4, 5). The outer membrane of gram-negative bacteria serves as an efficient permeability barrier to the inward flow of antibiotics (1). Nutrients and small hydrophilic molecules traverse this membrane through porins, which produce water-filled channels across the membrane (1, 9, 13). Gram-positive bacteria do not contain an outer membrane. The mycobacteria are gram positive. The cell wall peptidoglycan is covalently linked to arabinogalactan molecules that are esterified to mycolic acids. Although the molecular architecture of the mycobacterial cell wall is at present incompletely understood, it is predicted that the mycolates form a highly hydrophobic lipid layer (3). This lipid layer acts as a permeability barrier similar to the outer membrane of gram-negative bacteria. Permeability of small, hydrophilic molecules has been studied in *Mycobacterium chelonae* (6), *Mycobacterium smegmatis* (18), and *Mycobacterium tuberculosis* (4). Permeability coefficients of small molecules such as glucose, glycine, and leucine range from 17×10^{-8} to 620×10^{-8} cm/s in *M. chelonae* (6). These values are 1,000 to 10,000 times smaller than those of gram-negative bacteria such as *Escherichia coli*. Despite the low permeability of the mycobacterial cell wall, small hydrophilic molecules penetrate through water-filled channels of pore-forming proteins (19). One mycobacterial porin has been purified from *M. chelonae* (17). It is cation selective. We report here the purification of a pore-forming protein from *M. smegmatis*, which is widely used as a model system for genetic studies in *M. tuberculosis* (16) and for studies of the biological processes of mycobacteria, including diffusion of drugs through the cell wall (18). This porin allows the nonspecific permeation of small molecules such as sugars and amino acids.

Purification of a pore-forming protein. *M. smegmatis* SN₂ was grown for 48 h in Middlebrook 7H9 (Difco) broth supplemented with 0.05% Tween 80, diluted 10-fold into the same medium, and grown for 24 h in a rotary shaker at 37°C. Cells were collected by centrifugation, washed with 20 mM Tris-HCl buffer (pH 8), and stored at -70°C before use.

Cells were suspended in 20 mM Tris-HCl (pH 8) (buffer A) and disrupted by sonication for 15 min in 3-min bursts (Lab-sonic L; B. Braun, Melsungen, Germany). The cell wall was obtained as described by Trias and Benz (18). Briefly, the

homogenate was centrifuged at $1,500 \times g$ for 10 min, the supernatant was collected and pelleted at $45,000 \times g$ for 30 min, and the pellet was resuspended in buffer A, applied to a step gradient of 30, 40, and 70% sucrose, and centrifuged in a swinging bucket rotor at $100,000 \times g$ overnight. All steps were carried out at 4°C. The cell wall fraction was obtained at the interface between the 40 and 70% sucrose layers, washed twice with buffer A (centrifuged at $100,000 \times g$), and stored overnight at 4°C.

The cell wall fraction was washed once by centrifugation with buffer A containing 3 mM sodium azide and 1% Zwittergent 3-12 (Sigma) (buffer B). Proteins were extracted from the cell wall with buffer B containing 40 mM Na₂EDTA at room temperature for 1 h, followed by centrifugation at $100,000 \times g$ for 1 h. The pellet was further extracted with buffer B containing 0.5 M NaCl for 1 h at room temperature. After centrifugation at $100,000 \times g$ for 1 h, the supernatant was taken and dialyzed against buffer A containing 0.1% Zwittergent 3-12 (buffer C). The dialysate was applied to an anion-exchange (Resource Q; 1-ml) column equilibrated in the same buffer and coupled to a Pharmacia fast-performance liquid chromatography system. After the column was washed with the same buffer, proteins were eluted with a gradient of 0 to 1 M NaCl in buffer C. Fractions were dialyzed against 5 mM Tris-HCl (pH 8) containing 0.1% Zwittergent 3-12 (buffer D) and assayed for pore-forming activity by the liposome swelling technique described below. Fractions were concentrated by precipitation with chilled acetone and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7) with appropriate marker proteins (Bio-Rad).

The procedure used for sequential extraction of proteins from the cell wall with detergent was similar to that used by Trias and Benz (17, 18). However, while in their study the EDTA-containing extract was used to purify the porin, in the present study, no pore-forming activity was detected in this fraction. On the other hand, the NaCl-containing extract showed pore-forming activity after dialysis against buffer D. This fraction was therefore further purified through an anion-exchange column. The unbound fraction did not show any pore-forming activity. A pore-forming protein eluted near a concentration of 0.7 M NaCl and was homogeneous on SDS-PAGs (Fig. 1). It migrated as a 40-kDa polypeptide.

No difference was observed on SDS-PAGs when the protein was electrophoresed after incubation with gel denaturing buffer at 100 or 20°C for 30 min. It was not modified by heat (data not shown), as described for some porins of gram-negative bacteria (15).

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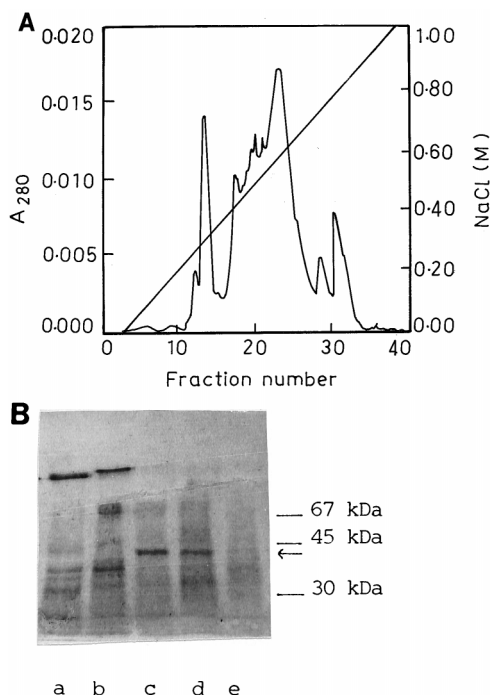


FIG. 1. (A) Fractionation of the *M. smegmatis* porin on a Resource Q column. Chromatography on Resource Q columns was performed after selective extraction of proteins from the cell wall of *M. smegmatis* as described in the text. Bound proteins were eluted with a gradient of 0 to 1 M NaCl in buffer. (B) SDS-PAGE analysis of proteins present in the crude cell wall (lane a) or fractions 30 (lane b), 31 (lane c), 32 (lane d), and 33 (lane e) of the Resource Q column. Two hundred microliters of each column fraction was concentrated by precipitation with chilled acetone and loaded on the gel. The arrow indicates the position of the 40-kDa protein.

Liposome swelling assay and pore-forming activity. Proteoliposomes were prepared as described by Nikaido and Rosenberg (12, 20). Briefly, a mixture of 2.4 μmol of acetone-washed egg phosphatidylcholine and 0.1 μmol of dicylphosphate was dried under a stream of nitrogen at the bottom of a test tube. The lipid was dissolved in anhydrous benzene and dried again to remove traces of water. It was then dissolved in diethyl ether and dried to produce a thin film. The drying process was completed by leaving the tubes for at least 2 h under vacuum over a desiccant. The film was suspended in 0.2 ml of buffer to which protein (50 μg of cell wall or 5 μg of column fraction) was added. The mixture was sonicated for 2 min in a bath-type sonicator and dried under vacuum. Finally, the film was reconstituted with 0.4 ml of a solution containing stachyose (Sigma) at a concentration which was isotonic for the above liposomes. The diffusion was studied in 5 mM Tris-HCl (pH 8) (8). Cell wall or detergent extract was dialyzed against buffer D prior to use in the liposome swelling assay. Specific activity is defined as the change in optical density $\times 1,000$ per minute per microgram of protein.

The diffusion of various uncharged molecules (saccharides) through the channel formed by the 40-kDa *M. smegmatis* porin was tested in liposome swelling assays. The rate of diffusion was inversely related to the size of the molecule tested (Fig. 2). The specific activity for arabinose diffusion into the proteoliposomes was 23. This value was lower than that reported for *Escherichia coli* (approximately 380) (11) or for protein F of *Pseudomonas aeruginosa* (approximately 97) (10). The pore diameter of the mycobacterial porin was calculated by comparing the diffusion rates of uncharged saccharides of different

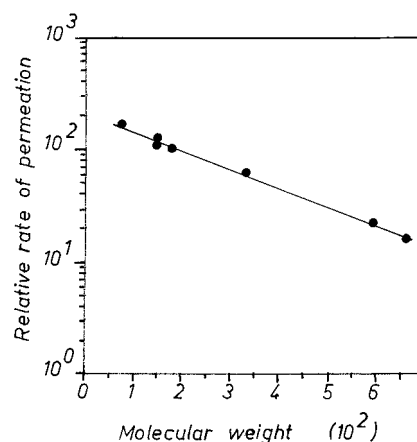


FIG. 2. Diffusion rates of small molecules in liposomes reconstituted with purified 40-kDa porin. Liposome swelling assays were performed as described in the text. The rates are expressed relative to the rate of permeation of glucose, which is set at 100. The following compounds were used (from top to bottom): glycine, arabinose, ribose, glucose, sucrose, raffinose, and stachyose.

molecular sizes measured by the liposome swelling technique and by applying the Renkin equation (11). From the relative diffusion rates and the hydration radii of the sugars (14), the pore diameter was calculated to be 2 nm. This value was similar to that reported for the *M. chelonae* porin by Trias et al. (19) and that formed by protein F of *P. aeruginosa* (20) but was much larger than those reported for *E. coli* OmpC (1.1 nm) and OmpF (1.2 nm) (9). However, on the basis of the Renkin equation and single-channel conductance, the channel diameter estimated with the strain *M. smegmatis* mc²155 by Trias and Benz is 3 nm (18).

N-terminal sequence analysis. Following SDS-PAGE, the purified protein was eluted from gel slices and subjected to N-terminal sequencing at the protein sequencing facility of Eurosequence (Groningen, The Netherlands) on an Applied Biosystems Pulsed Liquid Sequencer with on-line analysis of the phenylthiohydantoin amino acids. The sequence of the 15 N-terminal amino acids was determined to be M-N-K-A-E-L-I-D-V-L-T-T-X-M-G-T-. This sequence did not show any similarity with the known sequences of porins from other bacteria, most of which are enteric bacteria. This may be due to their different evolutionary origin. A single N-terminal sequence further confirmed the homogeneity of the purified porin.

The *M. smegmatis* porin (M_r , 40,000) is likely to be somewhat different from the *M. chelonae* porin (M_r , 59,000), since the two organisms are distantly related (19). The former porin is extracted from the cell wall in detergent-containing buffer supplemented with 0.5 M NaCl, while the *M. chelonae* porin is extracted in the same buffer supplemented with EDTA but not NaCl (17). However, the calculated pore diameter of 2 nm was similar to that reported for the *M. chelonae* porin (19). The channel formed by the 40-kDa porin allowed the nonspecific permeation of small hydrophilic molecules such as sugars and amino acids. Further knowledge about this porin and its possible involvement in the permeation of hydrophilic antibiotics could help in overcoming the permeability barrier of these bacteria with intrinsically low permeability toward hydrophilic molecules.

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