Purification and Characterization of Two Kinds of Porins from the Enterobacter cloacae Outer Membrane

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Two major outer membrane proteins of Enterobacter cloacae 206 were purified and identified as porins by using reconstituted vesicles. The 37-kilodalton porin forms a channel with a radius of 0.6 nm, which prefers positively charged substances to negatively charged ones, whereas the 39- to 40-kilodalton porin forms a larger channel with a radius of 0.8 nm, which has weaker selectivity for electric charges.

We have previously reported that Enterobacter cloacae 206 has major outer membrane proteins with masses of 37, 39, and 40 kilodaltons (K) and 39 to 40K, which were responsible for permeation of glucose and β-lactam antibiotics across the outer membrane (11). In this study, we purified these two proteins and identified them as porins using reconstituted vesicles containing these proteins.

The 37K and 39 to 40K proteins were isolated and purified from outer membrane mutants of strain 206, i.e., strains 206C1 and 206C3, which lacked the 39 to 40K and 37K proteins, respectively (12, 14). Strains 206C1 and 206C3 were cultured aerobically at 37°C for 18 h in L-broth and medium A (5) supplemented with 0.2% glucose and 0.1% Casamino Acids, respectively. Bacterial cells in the exponential growth phase were collected by centrifugation and disrupted in 0.05 M Tris-hydrochloride buffer (pH 7.2) by ultrasonication (cell disruptor 200; Branson Sonic Power Co.) for 2 min at 4°C. The crude membrane preparation was obtained by ultracentrifugation of the disrupted cell suspension at 100,000 × g for 30 min. The membrane proteins were solubilized from the membrane preparation with sodium dodecyl sulfate (SDS) solution with or without NaCl. The 37K and 39 to 40K proteins were selectively solubilized in the SDS solution containing 0.4 M NaCl. This procedure was essentially the same as that applied previously to the extraction of the Salmonella typhimurium porin (15). Salt-extracted proteins were purified by gel filtration by the method of Tokunaga et al. (14), except that a Sepharose CL6B column was used instead of a Sepharose 4B column. The purity of the 39 to 40K and 37K proteins was judged to be about 82% and more than 98%, respectively, by using SDS-polyacrylamide gel electrophoresis (11). The purified preparations were heated at 100°C in SDS solution before electrophoresis (Fig. 1).

To examine whether the 37K and 39 to 40K proteins form channels in a lipid membrane, we prepared phospholipid vesicles containing these proteins as follows. A mixture of 4 mg of E. cloacae phospholipids, which was extracted from the E. cloacae 206 cells and purified by the method of Bligh and Dyer (1), and 0.37 mg of cardiolipin (Sigma Chemical Co.) in a test tube was dried from chloroform solution to a thin film with nitrogen gas under reduced pressure for 2 h. Then, a solution containing 30 μg of the outer membrane protein was added. The preparation was briefly sonicated with a Branson water bath sonicator and dried to a thin film with nitrogen gas at 45°C under reduced pressure for 2 h.

One-half milliliter of 50 mM phosphate buffer (pH 7.0) containing 6% Dextran T-10 was added to the test tube, and the preparation was mixed with a Vortex mixer for 30 s to make vesicles.

Permeation rates of various sugars through the channels in the reconstituted vesicles were determined by the method of Luckey and Nikaido (4). The protein-containing vesicles were found to allow penetration of sugars by their molecular sizes (Fig. 2), whereas the penetration of sugars into the vesicles without the proteins was less than the limit of detection (data not shown). From these results, we identified the 37K and 39 to 40K proteins as porins. From the molecular weight dependence of sugar permeation rates, the radius of the pore formed by the 37K porin (37K protein) and the 39 to 40K porin (39 to 40K protein) were estimated to be 0.6 and 0.8 nm, respectively, by application of the Renkin equation (6, 10). The 37K porin channel has a radius similar to that of porin channels of E. coli, which were reported to be 0.58 nm (Omp F) and 0.54 nm (Omp C) by Nikaido and Rosenberg (7). However, the radius of the 39 to 40K porin channel is significantly larger than that of E. coli.

The effect of hydrophobicity and electric charge of solute on permeability through these channels was examined by measuring the permeability of eight cephalosporins into membrane containing porin- and lipopolysaccharide-containing vesicles enclosing a cephalosporinase purified from Citrobacter freundii GN346. Purification and details of the properties of the enzyme have been reported previously (12). A thin film of lipid containing E. cloacae phospholipids and cardiolipin was formed in a test tube by the procedure described above. Two milligrams of lipopolysaccharide extracted from E. cloacae 206 by the hot phenol extraction method (3) were added to the test tube with 0.5 ml of 5 mM MgSO4 solution, and the mixture was briefly sonicated in a water bath sonicator. Then, the mixture was lyophilized. To the preparation, 300 μg of E. cloacae porin solubilized in 300 μl of 50 mM phosphate buffer (pH 7.0) containing 0.167 M β-D-octylglucoside (Sigma) was added, and the mixture was stirred with a Vortex mixer and brief sonication until it became clear. Then, the cephalosporinase (11.72 nmol) was added to the solution, and the mixture was dialyzed against 50 mM phosphate buffer (pH 7.0) containing 5 mM MgSO4 to form vesicles. The cephalosporinase remaining outside the vesicles was irreversibly inactivated by 2.4 μmol of sulbactam, and then the vesicles were separated from free cephalosporinase and the inhibitor by gel filtration with a Sepharose CL6B column. Th assay of drug permeation was carried out by the method of Yamaguchi et al. (16). The hydrophobicity of cephalosporins was measured by reversed-phase thin-layer
chromatography (13) and expressed as $R_f$. A larger $R_f$ indicates a higher hydrophilicity of the molecule. As a control, the vesicles without porin were also prepared. Under our experimental conditions, the permeability of cephalosporins to the control vesicles was less than 20% of that of the porin-containing vesicles, even when the cephalosporins were moderately hydrophobic, because our vesicles contained lipopolysaccharide. Permeability of monomeric cephalosporins to both the 37K and 39 to 40K porin channels increased with increase of hydrophilicity, whereas the dependence of the permeability on hydrophilicity in the case of the 39K porin was somewhat larger than that of the 39 to 40K porin, although the permeability would not simply be proportional to the $R_f$ (Table 1). The deviation from the linearity of the dependency may indicate the presence of another factor affecting the permeability. Cephaloridine, which has an additional positive charge on its pyrimidine residue, showed an extremely accelerated rate of permeation for the 37K porin. However, cephaloridine did not show such a high permeation for the 39 to 40K porin, although its permeability for the 39 to 40K porin was obviously higher than that of monomeric cephalosporins similar in hydrophobicity to cephaloridine. The addition of an amino residue on the side chain at the 7-position of cephalosporins may not contribute to accelerating the permeability in the case of cefadroxil, whereas the permeability of cephalixin, which has a moderately hydrophilic property, was relatively higher than that assumed on the basis of its low hydrophilicity, which is probably due to the amino residue. The function of amino residues as a source of positive charge may be weak in a neutral solution, because the $pK_a$ of amino residues of cephalixin and cefadroxil is 7.3 (2) and 7.4 (personal communication, laboratory of Banyu Pharmaceutical Co., Tokyo, Japan), respectively. An additional negative charge at the 7-position of cephalosporin-C depressed the permeability of this antibiotic for the 37K porin. However, the permeability of cephalosporin-C for the 39 to 40K porin was about the

**TABLE 1. Relative permeability of cephalosporins into the porin-containing vesicles**

<table>
<thead>
<tr>
<th>Drug$^a$</th>
<th>Mol wt</th>
<th>Dissociable residues$^b$</th>
<th>$R_f$</th>
<th>Relative permeability$^c$ of the following porins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39 to 40K 37K</td>
</tr>
<tr>
<td>CTZ</td>
<td>416</td>
<td>C</td>
<td>0.87</td>
<td>1.73 2.08</td>
</tr>
<tr>
<td>CEC</td>
<td>338</td>
<td>C</td>
<td>0.86</td>
<td>1.04 1.66</td>
</tr>
<tr>
<td>CEZ</td>
<td>431</td>
<td>C</td>
<td>0.81</td>
<td>1.00 1.00</td>
</tr>
<tr>
<td>CET</td>
<td>395</td>
<td>C</td>
<td>0.46</td>
<td>0.71 0.62</td>
</tr>
<tr>
<td>CDX</td>
<td>387</td>
<td>C, A</td>
<td>0.89</td>
<td>0.96 2.02</td>
</tr>
<tr>
<td>CEX</td>
<td>348</td>
<td>C, A</td>
<td>0.57</td>
<td>1.04 1.37</td>
</tr>
<tr>
<td>CER</td>
<td>415</td>
<td>C, P</td>
<td>0.39</td>
<td>1.42 4.75</td>
</tr>
<tr>
<td>CEP-C</td>
<td>414</td>
<td>C, C, A</td>
<td>0.92</td>
<td>2.00 0.72</td>
</tr>
</tbody>
</table>

$^a$ Abbreviations: CTZ, ceftizole; CEC, cepacetrile; CEZ, cefazolin; CET, cefalotin; CDX, cefadroxil; CEX, cephalixin; CER, cephaloridine; CEP-C, cephalosporin-C.

$^b$ C, A, and P, carboxyl, amino, and pyrimidine residues, respectively.

$^c$ Permeability was normalized to that of cefazolin.
same as that of monoanionic cephalosporins, which show similar hydrophilicity. Such characteristics of the 39 to 40K porin were apparently similar to those of the PhoE porin of *E. coli*. The PhoE porin channel was reported to show less preference to positive charge than the OmpF and OmpC channels and no denial for negative charge (8). The fixed charge on the PhoE porin channel is considered to contribute to these characteristics (9). However, these characteristics of the 39 to 40K porin channel may be attributable to weak interaction between solute and the wall of the channel due to its large pore size. In this study, we demonstrated that *E. cloacae* 206 has two kinds of porins which differ from one another in the pore size and the behavior toward the solutes that permeate through the channels formed by them. It is interesting to know the physiological role of these two kinds of porins in the organisms.

**LITERATURE CITED**