Identification of Porins in the Outer Membrane of *Pseudomonas aeruginosa* That Form Small Diffusion Pores*

Eisaku Yoshihara and Taiji Nakae
From the Department of Cellular Information Sciences, Tokai University School of Medicine, Isehara 259-11, Japan

The purified outer membrane proteins of *Pseudomonas aeruginosa* were reconstituted with phosphatidylcholine and dicetylphosphate into membrane vesicles, and these were tested by the liposome swelling method for the diffusion of saccharides with different *M*ₐ. Proteins C (*M*ₐ, 70,000), D (*M*ₐ, 46,000), and E (*M*ₐ, 43,000) were found to confer the monosaccharide-permeable pores in the reconstituted liposome membranes. The membrane vesicles containing proteins F (*M*ₐ, 34,000), G (*M*ₐ, 25,000), or H (*M*₂, 19,000) showed no detectable pore-forming activity. The pores formed by proteins C, D, or E appeared to be smaller than that formed by the *Escherichia coli* porins. The size of the solutes that permeated through the newly identified porins is similar to that through the intact and purified outer membranes of *P. aeruginosa* (Yoneyama, H., and Nakae, T. (1986) *Eur. J. Biochem.* 157, 33-38; Yoshihara, E., Gotoh, N., and Nakae, T. (1988) *Biochem. Biophys. Res. Commun.* 156, 470-476).

*Pseudomonas aeruginosa* is a pathogen to the immunocompromised or cystic fibrosis patients and shows a high intrinsic resistance against a number of structurally unrelated antibiotics (1). One of the important factors contributing to this drug resistance has been thought to be the diffusion barrier at the outer membranes (2, 3). It was reported, however, that protein F in the outer membrane of *P. aeruginosa* forms a large diffusion pore that allows the diffusion of the polysaccharides with *M*ₐ up to 7,000 (4), but the majority of the pores are nonfunctional (5, 6). On the other hand, Caulcott et al. (7) and Yoneyama et al. (8, 9) presented lines of evidence that the intact outer membrane of *P. aeruginosa* is only permeable to the saccharides that are smaller than about di- or trisaccharides as measured by the equilibrium of test solutes. Proteoliposomes were reconstituted from the purified outer membranes and phospholipids containing either stachyose (*M*ₐ, 666) or dextran T-10 (*M*₂, 10,000). The relative diffusion rates of mono- and oligosaccharides and the apparent exclusion limits, *M*ₐ, about 250, in these proteoliposomes were indistinguishable from each other as determined by the liposome swelling assay (10). The proteoliposomes reconstituted from the protein F-sufficient or -deficient outer membranes showed indistinguishable diffusion rates and apparent exclusion limits (10). These results indicated that the outer membrane of

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* Portions of this paper (including "Experimental Procedures" and Figs. 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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2. The abbreviations used are: C₃₂H₆₆O₂₄, octaethylene glycol dodecyl ether; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PC, phosphatidylcholine; DCP, dicetylphosphate; MOPS, 3-(N-morpholino)propanesulfonic acid.
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Pore-forming activity of the purified outer membrane proteins

Outer membrane proteins were purified from P. aeruginosa PA01 as described under “Experimental Procedures.” Liposomes were reconstituted from 5 μg of the purified outer membrane proteins and 1 μmol of PC/DCP (97/3, molar ratio) as described under “Experimental Procedures.” The diffusion rate of arabinose was calculated from the rate of proteoliposome swelling as described earlier (17, 20). Relative diffusion rates were expressed as percentages against the rate through protein E. The values are the mean ± S.D. of more than three independent assays.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Apparent Mr</th>
<th>Diffusion rates</th>
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<tbody>
<tr>
<td>C</td>
<td>70,000</td>
<td>0.0549 ± 0.0051</td>
</tr>
<tr>
<td>D</td>
<td>46,000</td>
<td>0.0974 ± 0.0239</td>
</tr>
<tr>
<td>E</td>
<td>43,600</td>
<td>0.1358 ± 0.0103</td>
</tr>
<tr>
<td>F</td>
<td>34,000</td>
<td>0.0057 ± 0.0051</td>
</tr>
<tr>
<td>G</td>
<td>23,000</td>
<td>0.0023*</td>
</tr>
<tr>
<td>H</td>
<td>19,000</td>
<td>0.0015</td>
</tr>
</tbody>
</table>

*This value is the mean of two independent assays.

Table I

FIG. 3. Effect of the amount of protein E on the diffusion rate of arabinose. Proteoliposomes were reconstituted from appropriate amounts of protein E and 1 μmol of phospholipids (PC and DCP in 97/3 molar ratio) as described under “Experimental Procedures.” The diffusion rate of arabinose through these proteoliposomes was determined by the liposome swelling assay as described under “Experimental Procedures.”

increased (Fig. 3), indicating that the diffusion rate depends on the amount of porin added. Similar results were obtained for proteins C and D (data not shown). However, the diffusion rates were not strictly proportional to the amounts of protein added as observed elsewhere (16). This result showed that solute diffusion through P. aeruginosa pores is very inefficient compared with the Escherichia coli porin (17, 18).

In order to characterize the pores formed by these newly identified porins, the diffusion rates of uncharged saccharides with different Mr were determined, and the results were plotted according to the method of Renkin (19) and that of ours (20) (Fig. 4). The slope of the plots of log relative diffusion rate versus Mr appeared to be −2.6, −3.2, and −3.7 for proteins C, D, and E, respectively. These results indicated that the size of pores made of proteins D and E are very similar and they are slightly smaller than the pore made of protein C. The sizes of pores made by these P. aeruginosa porins appeared to be smaller than those of the E. coli B porin, since the slope of the plots for the E. coli B porin appeared to be −1.7.

Newly identified porins C, D, and E seem to be relatively unstable in the surfactant solution. When proteins C and D were kept in a solution containing 10 mM Tris-HCl, 34 mM β-octyl glucoside, and 1 mM EDTA at 4°C for 1 month, a significant reduction in the pore-forming activity was observed. Furthermore, the pores of such porins seemed to be dilated, since the reconstituted membranes became more permeable to sucrose compared with the freshly prepared porins. Though protein E seems to be a little more stable than the other two, prolonged storage over a period of a month under the above conditions cannot be recommended. Because of these observations, the authors are not certain whether a slightly large size (slope = −2.6) of protein C pore compared with that of protein D and E (slopes = −3.2 and −3.7, respectively) was characteristic of protein C or was due to such artifacts.

Since protein D1 was reported to be the glucose-inducible porin forming a pore of a similar size to proteins C, D, and E (21, 22), we examined the identities of porin D and protein D1. The outer membranes of P. aeruginosa were prepared from cells grown in glucose containing minimal medium with or without casamino acid, and the purified outer membranes were subjected to SDS-PAGE. The protein profiles were compared with a band of purified protein D run under the identical conditions. Fig. 5 showed that the positions to where proteins D1 and D traveled were proximal and distal, respectively, to the top of the gel. The result demonstrated that protein D is a distinct polypeptide from protein D1 and it is probably protein D2 (23). Protein D1 might not be induced under the present culture conditions, since it was reported that protein D1 was not detectable in the cells grown in L broth (21).

DISCUSSION

This paper reports that proteins C, D, and E are porins in the outer membrane of P. aeruginosa. The pores made by these porins are small as only solutes with less than about 250 can penetrate freely as assayed by the liposome swelling method. This observation is fully consistent with reports from our and other laboratories (7–10) but does not favor the earlier conclusion that protein F is a porin that forms a large diffusion pore (4, 5, 24). Although the reason for this discrepancy is not clear now, the following lines of evidence favor the present conclusion. (i) The presence of small pore(s) in the intact outer membrane was confirmed by the two independent groups using different techniques (7–9). (ii) Proteoliposomes reconstituted with the purified outer membrane and the surfactant solubilized outer membrane proteins preserved identical permeability properties as the intact outer membrane (10). (iii) Studies in other laboratories supported the above results. When the imipenem-resistant P. aeruginosa were selected from the imipenem-treated patients or in vitro, protein D was found to be missing (25–28). It was reported that protein D1 forms glucose- and xylose-selective pores but is
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Fig. 4. Permeability properties of the liposome membranes reconstituted from the newly identified porins. Proteoliposomes were reconstituted from 1 μmol of phospholipids (PC and DCP in 97/3 molar ratio) and 5 μg of the purified proteins C, D, or E as described under "Experimental Procedures." The diffusion rate of saccharides with various M_r through proteoliposomes were examined by the liposome swelling assay as described under "Experimental Procedures." Data were plotted according to the procedure of Renkin (19) for proteins C (A), D (B), and E (C) and our method (20) for proteins C (a), D (b), and E (c). Saccharides used were 1, arabinose; 2, ribose; 3, glucose; 4, galactose; 5, mannose; 6, α-methylglucoside; 7, α-methylmannoside; 8, N-acetylglucosamine; 9, sucrose; and 10, maltose. Data are mean of more than three independent assays. Broken lines show the results obtained with the liposomes reconstituted from the E. coli porin.

The reason why previous investigations did not discover the small pores is not clear to us. However, we interpret the earlier data as follows. (i) Most of the earlier investigators except those in Ref. 31 used sucrose as the smallest test solute (4, 5, 30), which was found to be barely permeable through the P. aeruginosa outer membrane. Thus, the investigators had less chance to discover the small diffusion pores. (ii) In the reconstitution experiments, the Triton X-100-solubilized membrane proteins were treated with ethanol overnight and dialyzed against water over a period of a month (24). Such treatment might cause denaturation and/or dilation of the

not selective for imipenem (22). The mutant strains of P. aeruginosa resistant to β-lactams, new quinolones, and chloramphenicol were missing proteins C, D, and/or E. The transduction of wild gene to these mutants restored these missing outer membrane proteins and antibiotic sensitivity partially. Amino acid sequence of protein F deduced from the nucleotide sequence of the gene coded for protein F showed no homology with the amino acid sequence of E. coli porins, Omp F, Omp C, Lam B, or Pho E. Instead, protein F showed the highest homology with Omp A of the E. coli outer membrane (29).

Y. Yamano, T. Nishikawa, and Y. Komatsu, personal communication.
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Fig. 5. Comparison of the outer membrane proteins in the cells grown in the presence or absence of glucose. *P. aeruginosa* PA01 was grown in BM 2 medium containing 0.4% of glucose with or without 0.2% of casamino acids as described in Ref. 21. The outer membranes were purified as described under “Experimental Procedures” and were subjected to SDS-PAGE (14% acrylamide) after heating at 95°C for 5 min in the solubilization buffer (53). Lane A, M, markers: carbonic anhydrase (29,000); egg albumin (45,000); bovine albumin (66,000); phosphorylase b (97,400); and β-galactosidase (116,000); Lane B, outer membrane proteins from the cells grown in the medium containing glucose without casamino acids; Lane C, outer membrane proteins from the cells grown in the medium containing glucose and casamino acids; Lane D, purified protein D. The arrowhead indicates the location of protein D.

pores as mentioned above. (iii) In the conductivity measurements, the investigators purified protein F by the procedure described in Ref. 24. It is known that the conductivity measurement is highly sensitive to the contamination of a small amount of pore-forming proteins and is insensitive to the presence of protein(s) forming a low conductivity pore (32).

Studies on the permeability of antimicrobial agents through membranes reconstituted from the purified proteins C, D, or E are a promising approach for revealing the characteristics of the intrinsic drug resistance of *P. aeruginosa*, and such studies are in progress in this laboratory.

REFERENCES

SUPPLEMENTAL MATERIAL TO
IDENTIFICATION OF PORINS IN THE OUTERMEMBRANE OF PSEUDOMONAS AERUGINOSA THAT FORM SMALL DIFFUSION PORES
EISAKU YOSHINARA and TAIJI NAKAE

EXPERIMENTAL PROCEDURES

Medium and Growth Conditions. P. aeruginosa PA01 and KG1079 (12) cells were grown in 1 L of the medium containing 10 g of Bacto-tryptone, 1 g of Yeast-extract, 5 g of NaCl, and 5 mM of MgCl₂, pH 7.4 at 37°C for 4 hr under vigorous aeration (200 rpm) after dilution of 100 ml of a fully grown preculture. The outer membrane was isolated according to the method of Mizuno and Kageyama (11).

Solubilization and Purification of the Outer Membrane Proteins. The outer membrane (100 mg of protein) was mixed with 5 ml of 10 mM Tris·HCl buffer, pH 8, containing 6 M dodecylether IC12E8 and 1 mM EDTA and subjected to sonic oscillation for 2 min in a bath-type sonicator (Bransonic B12). The centrifuged supernatant (100,000 xg for 30 min at 20°C) contained more than 95% of the outer membrane proteins. Supernatant was applied onto a DEAE ion-exchange HPLC column (TSKgel DEAE-5PW, 7.5 x 0.75 cm I.D.) equilibrated with 10 mM Tris·HCl buffer, pH 8, containing 5 mM octaethylene glycol dodecylether (C12E8) and 1 mM EDTA (Buffer A) and the column was eluted with a 0 to 0.5 M linear gradient of NaCl in Buffer A. Absorption at 280 nm was monitored and proteins were analyzed by SDS-polyacrylamide gel electrophoresis (10% acrylamide gel). M₆ markers for SDS-PAGE were obtained from Sigma. Protein was quantified by the method of Lowry et al. (13).

Proteins E (Mr, 43,000), G (Mr, 25,000) and H (Mr, 19,000) flowed through the column without adsorption. This flow-through fraction contained the high-porin activity protein as tested by the liposome swelling assay (pool 1). Then the column was eluted with a linear gradient of 0 to 0.5 M NaCl in Buffer A. The elution profile is shown in Fig. 1. Proteins C (Mr, 10,000), D (Mr, 46,000) and a small amount of F (Mr, 34,000) was eluted at a NaCl concentration around 0.2 M. This fraction also showed a high porin activity (pool 2). Pool 1 was concentrated using an ultrafiltration (Amicon PM10) and mixed with 1 mM MgCl₂. This material was applied again onto a DEAE ion-exchange HPLC column equilibrated with 10 mM Tris·HCl buffer, pH 8, containing 5 mM C12E8 and 1 mM MgCl₂ and the column was eluted with a 0 to 0.3 M linear gradient of NaCl in the same buffer. A homogeneous preparation of protein E was obtained in the flow-through fraction. Proteins G and H were eluted at a NaCl concentration around 0.1 M. Pool 2 was subjected to DEAE ion-exchange HPLC chromatography under identical conditions to the first chromatography in the presence of EDTA and the fractions enriched with proteins C and D, but reduced amounts of protein F were obtained at a NaCl concentration of around 0.15 M. This fraction was then applied onto a DEAE ion-exchange HPLC column equilibrated with 10 mM Tris·HCl buffer, pH 8, containing 34 mM p-octylglucoside and 1 mM EDTA. When the column was eluted with a 0 to 0.3 M NaCl gradient, protein D was eluted at around 0.14 M NaCl to separate lately eluted protein C.

By these procedures proteins C, D and E were purified to apparent homogeneity as judged by SDS-PAGE (Fig. 2). Protein F was also purified to near homogeneity, but the preparation was contaminated with a small amount of other proteins (Fig. 2). Proteins C and D could be purified without interference from protein F, when the outer membrane from protein F-deficient mutants e.g. KG1079 (12) was used and the DEAE ion-exchange HPLC chromatography was run in the presence of p-octylglucoside as described above.

Preparation of the Proteoliposomes and Permeability Assay. An appropriate amount of the purified proteins and 1 μ mole of a lipid mixture consisting of egg-yolk phosphatidylcholine (PC, Type V-E, Sigma) and dicetylphosphate (DCP, Sigma) (97/3, molar ratio) were dissolved in 100 μl of 68 mM p-octylglucoside and the mixture was subjected to sonic oscillation for one min in a bath-type sonicator. 0.2-2 Bio-beads (ca. 159 mg) was added and incubated for 4 hr with a light agitation, resulting in the spontaneous formation of proteoliposomes (14). The liposome suspension was dialyzed against a large excess of deionized water (Milli Q, Millipore Corp.) for 1 to 2 days by changing the dialysis water several times. Liposomes were dried under a N₂ gas stream followed by desiccation in an evacuated desicator for at least 1 hr. The dried materials were suspended in 133 μl of 40 mM stachyose in 1 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-NaOH buffer, pH 7.2 by mixing on a Vortex-Genie mixer at the highest speed for 20 sec. The permeability of the proteoliposomes to uncharged saccharides was examined by the liposome swelling assay as described earlier (15). The liposome membranes reconstituted by this method without protein are not permeable even to p-glucose.