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Purification and Properties of *Pseudomonas aeruginosa* Porin*

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Porin, a protein producing water-filled diffusion channels across membranes, was purified to homogeneity by differential extraction of *Pseudomonas aeruginosa* outer membrane or cell envelope, followed by gel filtration in cholate or dodecyl sulfate. Both the cholate- and dodecyl sulfate-purified porins were as active as the porin contained in the fragments of outer membrane in increasing the permeability of the liposome membranes reconstituted from these components and egg phosphatidylcholine. However, the *P. aeruginosa* porin was very different from the porins of *Escherichia coli* and *Salmonella typhimurium* at least in three aspects. (*a*) When the rates of the penetration of various sugars through the porin channels were determined by following turbidimetrically the osmotic swelling of reconstituted liposomes, we found that diffusion rates through *P. aeruginosa* porin channel were affected much less by the size of the solutes than were the rates through *E. coli* porin channels. These results indicated that the pore can be approximated as a hollow cylinder of about 1.0-nm radius, about 70% larger than the *E. coli* porin channel. (*b*) The permeability to small sugars produced by reconstituting a given amount of *P. aeruginosa* porin with phospholipids was about 40-fold lower than that obtained by the reconstitution of the same amount of *E. coli* OmpF porin. Together with results reported from another laboratory (Benz, R., and Hancock, R. E. W. (1981) *Biochim. Biophys. Acta* 646, 298–308), the results suggest that only a small fraction of the *P. aeruginosa* porin channel is "open" at any given time. (*c*) In contrast to *E. coli* porin whose only known active form is a trimer, the *P. aeruginosa* porin appears to exist as a functionally active monomer of 35,000 daltons in the presence of sodium dodecyl sulfate.

Pseudomonas aeruginosa is resistant to a wide range of antibiotics and other deleterious agents (1, 2), and it has been inferred that this property is related to the poor permeability of its outer membrane. In a previous study (3), we showed that the outer membrane of this organism indeed had permeability that was 10–100-fold lower than that of *Escherichia coli* outer membrane to a variety of solutes. A similar conclusion was drawn by a more limited comparison of the permeability of *P. aeruginosa* outer membrane to nitrocefin with that of *E. coli* outer membrane to other cephalosporins (4). On the other hand, the porin of *P. aeruginosa* was found to produce channels of relatively large size in reconstitution assays (5, 6). Benz and Hancock (6) observed that the number of open pores/unit area of black lipid membrane found after the addition of *P. aeruginosa* porin to the aqueous phase was about 2 orders of magnitude lower than the number of pores formed from *E. coli* porin under similar conditions. However, this could have been a reflection of the kinetics of insertion of porin molecules into the bilayer, rather than that of the intrinsic properties of the pores. Furthermore, in previous studies (5) it was difficult to purify the porin to homogeneity in a functionally active form. In this study, we describe methods for the purification of *P. aeruginosa* porin to homogeneity in a functional form, present evidence that the porin has an intrinsic property to produce channels of low permeability albeit with larger diameter, and show that the active preparation consists of monomers of the 35,000-dalton polypeptide, unlike in *E. coli* or *Salmonella typhimurium* where only trimers or higher aggregates are the active species (7, 8).

MATERIALS AND METHODS

Bacterial Strains and Their Cultivation—*P. aeruginosa* PAO1 and K799 were used (3); they were usually grown in L broth (1% Difco Tryptone, 1% Difco yeast extract, and 0.5% NaCl) at 37 °C with aeration by shaking. Cells were harvested at the late exponential phase by centrifugation at room temperature. Outer membranes were prepared as described previously (9).

Purification of Porin in Cholate—Outer membrane fraction obtained from 4 liters of a late exponential culture of *P. aeruginosa* was suspended in 20 ml of 2% sodium cholate, 0.2 M NaCl, 50 mM Tris-Cl, pH 8.0. After 20 min at room temperature, the suspension was centrifuged at 125,000 × *g* for 30 min. The pellet was then extracted with 5 ml of 2% sodium cholate, 1 M NaCl, 50 mM Tris-Cl, pH 8.0, 10 mM EDTA with brief sonication, and the mixture was centrifuged as above. Ammonium sulfate was added to the supernatant up to 35% saturation, and the salted out proteins were removed by centrifugation. The supernatant solution was applied to a column of Sephacryl S-200 (1.5 × 91 cm) equilibrated with 1% sodium cholate, 1 M NaCl, 10 mM Tris-Cl, pH 8.0, and the column was eluted with the same buffer. Porins were eluted as a broad peak soon after the void volume. Porin-containing fractions were combined and concentrated to 1.7 ml by dialysis against Ficoll 400, and the sample was fractionated again on the same column. The fractions with the highest degree of purity were pooled and dialyzed at 4 °C against 10 mM Tris-Cl, pH 8.0, to remove most of the NaCl and cholate.

Purification of Porin in Dodecyl Sulfate—Cells from 4 liters of late exponential culture were washed once and were suspended in 40 ml of 15 mM Tris-HCl buffer, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride, containing about 1 mg each of pancreatic deoxyribonuclease and ribonuclease, and the suspension was passed through a French pressure cell at 15,000 p.s.i. three times. After removing large debris by centrifuging at 3,000 rpm for 10 min, the crude envelope fraction was obtained by centrifugation at 100,000 × *g* for 45 min. The envelope fraction was extracted three times with 45 ml of 2% LDS[1], 15 mM Tris-HCl, pH 8.0, at 0 °C, each time followed by centrifugation

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[1] The abbreviations used are: LDS, lithium dodecyl sulfate; SDS, sodium dodecyl sulfate; LPS, lipopolysaccharides.

at 100,000 × *g* for 45 min at 4 °C. These extraction steps removed all cytoplasmic membrane proteins and most of the outer membrane proteins, leaving behind the complex of peptidoglycan sheets with the porin (protein F (10)) and protein H (10) (see also Ref. 11). Finally, the pellet was suspended in 3 ml of 2% LDS, 1 M LiCl, 15 mM Tris-HCl, pH 8.0, and the suspension was incubated at 37 °C for 90 min. Centrifugation as above produced a supernatant containing essentially only porin and protein H. This extract was applied to a column of Sephacryl S-200 (1.5 × 91 cm) and was eluted with 1% LDS, 0.5 M LiCl, 10 mM Tris-Cl, pH 8.0, or with 1% SDS, 0.4 M NaCl, 10 mM Tris-Cl, pH 8.0, at room temperature. The porin was eluted as a nearly symmetrical peak at the K_d value of about 0.15, followed by the trailing peak of protein H. The purity, judged by the scanning of the stained SDS-polyacrylamide gel, was at least 98% (see Fig. 1).

Liposome Swelling Assay—This was carried out essentially as described earlier (12) but usually by using a mixture of acetone-extracted egg phosphatidylcholine and dicetylphosphate as the phospholipid (13). For some experiments, phospholipids and LPS from *P. aeruginosa* were added; these were prepared from strain PAO1 cells by the methods of Folch *et al.* (14) and Westphal *et al.* (15), respectively. The porin preparation purified in dodecyl sulfate was usually dialyzed briefly against 0.1 M NaCl, 10 mM Tris-Cl, pH 8.0, in order to remove much of the NaCl. The dodecyl sulfate carry-over from the porin preparation (usually less than 50 μg in a mixture containing 2 μmol of phospholipids) did not affect the results. Preliminary experiments showed that 120 μg of SDS can be added to 2 μmol of phospholipids without a noticeable increase in nonspecific permeability of the liposome membrane; previously, it was shown that 140 μg of Triton X-100 could be added to 1 μmol of phospholipids without any undesirable effect (5).

The extent of incorporation of the added porin into the liposome bilayer was examined as follows. Egg phosphatidylcholine (10 μmol) and dicetylphosphate (0.2 μmol) were dried as a film at the bottom of a test tube, and 50 μg of *P. aeruginosa* porin were added. Liposomes were made according to the standard procedure (13), *i.e.* by drying the sonicated porin/phospholipid mixture under reduced pressure and resuspending the mixture in 0.5 ml of 10 mM Tris-HCl buffer, pH 7.4. The liposome suspension was mixed with 1.5 ml of 67% (w/v) sucrose in 10 mM Tris-HCl buffer, pH 7.5, and the mixture was transferred to a centrifuge tube. Two additional layers, one containing 2 ml of 33% (w/v) sucrose and the other containing 1 ml of 10 mM Tris-HCl buffer, were added, and the tubes were centrifuged at 35,000 rpm in a Beckman SW 65 rotor for 18 h at 20 °C. Practically all of the liposomes floated to the interface between the buffer and the 33% sucrose layer, and protein assay in the presence of excess SDS (16), using as control the corresponding fractions from a control gradient containing liposomes without porin, showed that at least 90% of the added porin was associated with liposomes. Another control experiment with the porin alone in the bottom layer showed that the porin by itself could not float up to this interface.

Analytical Methods—Protein was usually determined by the Lowry method (17), with bovine serum albumin as standard. For the assay of *P. aeruginosa* porin used in the sedimentation equilibrium experiment, the protein was hydrolyzed in 6 N HCl at 110 °C for 20 h, the amino acid contents were determined with a Beckman amino acid analyzer, and the amount of protein was calculated from the observed amounts of five more abundant amino acids and the reported amino acid composition (11) of this protein. The amount of SDS bound to the protein was determined as described by Yu *et al.* (18). SDS-polyacrylamide electrophoresis was performed as described by Lugtenberg *et al.* (19); pretreatment of the samples with phenol (9) was not carried out.

Protein Cross-linking—Porin or envelope fractions in 0.2% SDS, 5 mM sodium phosphate buffer, pH 8.0 (protein concentration 1–2 mg/ml), were mixed with an equal volume of 1 M triethanolamine-HCl buffer, pH 8.5, and 50 μmol of dimethylsuberimidate (Sigma) were added to 1 ml of this mixture. The solution was kept at room temperature for 1 h, the excess dimethylsuberimidate inactivated by adding either methylamine or Tris, and the mixture was dialyzed against 0.2% SDS, 5 mM sodium phosphate buffer and then was analyzed by SDS-polyacrylamide gel electrophoresis.

RESULTS

Purification of P. aeruginosa Porin

In a previous work (5), porin (or protein F according to the nomenclature proposed in Ref. 10) was purified by ion exchange chromatography of Triton X-100 extracts of outer membrane. Although this procedure resulted in a considerable purification of porin, it was usually difficult to obtain a homogeneous porin preparation by this method (5). We, therefore, sought to find methods that produced homogeneous porin preparations easily and reproducibly.

The first method used differential extraction of the outer membrane in cholate and gel filtration in this detergent. As shown in Fig. 1, extraction in cholate containing 0.2 M NaCl removed most of the non-porin proteins, and subsequent extraction in cholate containing 1 M NaCl plus 10 mM EDTA produced a preparation somewhat enriched in porin. Two rounds of gel filtration on Sephacryl S-200 produced an essentially pure porin preparation.

We then noticed that *P. aeruginosa* porin retained its pore-forming function in SDS or in LDS. Thus, the method based on a differential extraction of crude cell envelope fraction in LDS followed by gel filtration was established (see "Materials and Methods") (Fig. 1). The preparation retained its channel-forming activity fully at room temperature for at least 2 weeks; on SDS-polyacrylamide gel electrophoresis, these preparations produced a single band at the position of F-protein that corresponds to the apparent molecular weight of 36,000. Similar stability was observed upon dialysis of the preparation against 5 mM Tris-Cl buffer containing 0.1% SDS and 0.1 M NaCl. However, dialysis against water or low ionic strength buffer always resulted in the partial conversion of the material into the form that migrated at the F* position (11), corresponding to the apparent molecular weight of 40,000, with the

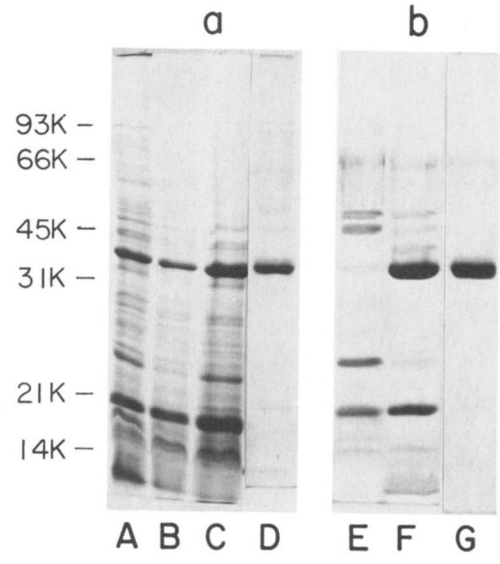

FIG. 1. **Purification of *P. aeruginosa* porin.** SDS-polyacrylamide gel electrophoresis patterns of some fractions are shown. *a*, purification in cholate (*lanes A–D*). Outer membrane fraction from strain PAO1 (*lane A*) was first extracted with 2% sodium cholate, 0.2 M NaCl and then 2% sodium cholate, 1 M NaCl (extract shown in *lane B*). Some contaminating proteins were precipitated with ammonium sulfate, and the supernatant (*lane C*) was purified by two successive steps of gel filtration. The central fraction of "porin peak" in the second gel filtration step was essentially pure as shown in *lane D*; the purity, determined by the scanning of the gel to which about 10 μg of protein were applied, was 97%. *b*, purification in dodecyl sulfate (*lanes E–G*). Many envelope proteins were extracted by LDS at 4 °C (*lane E* shows the first extract), leaving behind peptidoglycan layers with two major noncovalently associated proteins, F and H (*lane F*). Most of porin (F-protein) and some of protein H were extracted by LDS containing 1 M LiCl (not shown), and two gel filtration steps resulted in the elution of fractions, some of which were at least 99% pure on the basis of scanning of the gel (*lane G*). *93K*, 93,000 daltons, for example.

concomitant decrease in the activity to form channels in the reconstitution assay. Precipitation of this dialyzed porin preparation with trichloroacetic acid produced a complete conversion to the F*-form, with the total loss of channel-forming activity. This apparent denaturation in low ionic strength media containing dodecyl sulfate may explain the previous failure in our laboratory to obtain functionally active *P. aeruginosa* porin in SDS (20). F to F* conversion was found to be caused by treatment with hot aqueous phenol by Hancock and Nikaido (9) and also prolonged heating as well as precipitation with trichloroacetic acid by Mizuno and Kageyama (11); the latter authors also found that the conversion was accompanied by a change in circular dichroism spectra from that rich in β-sheet structure to that rich in α-helix and random coils.

Channel-forming Activities of the Porin Preparations

The channel-forming activities of various preparations were determined by reconstitution into liposomes (see "Materials and Methods"). Several significant observations may be made from these experiments.

First, the dependence of permeability on the size of neutral solutes (sugars) was far less steep than with the *E. coli* porins (Fig. 2). Because the limited permeability of larger solutes is likely to occur as a consequence of the collision of the solutes with the rims of the pore (see Ref. 21), this result supports the previously advanced notion (5) that the *P. aeruginosa* porin channel has a significantly larger diameter than the *E. coli* porin channels.

Second, the degree of permeability (toward glucose or arabinose) produced by the incorporation of a given amount of *P. aeruginosa* porin was always much less than what was produced by the incorporation of an equivalent amount of *E. coli* OmpF porin, usually by a factor of about 40 (see Fig. 3). This was not due to the inactivation of porin during purification, as the porin preparation purified in LDS had the same activity as that purified in a "mild" detergent such as cholate, and even fragments of outer membranes or whole envelope fractions showed identical activity when the results were normalized to the amounts of porin present in these preparations (Fig. 3). Nor can the results be explained by inefficient incorporation of porin into liposome membranes, as the sucrose density gradient flotation experiment (see "Materials and Methods") showed that at least 90% of the porin added was associated with liposomes.

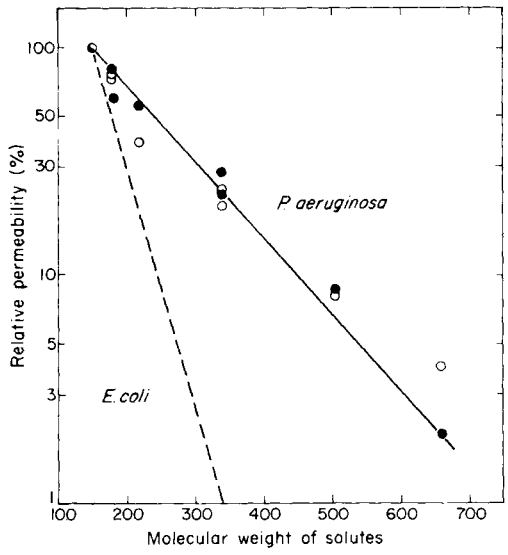

FIG. 2. **Permeability of liposome reconstituted from *P. aeruginosa* porin or crude envelope fraction and phospholipids.** *P. aeruginosa* PAO1 porin purified in cholate (1.5 μg of protein) (●) or the envelope fraction containing 2 μg of porin (○) was added to 2.25 μmol of egg phosphatidylcholine, and liposomes containing 17% (w/v) dextran T-20 were prepared (see "Materials and Methods"). The liposomes were diluted into isotonic (about 40 mM) solutions of a sugar, and permeability was determined from the initial rate of change of optical density of liposomes, measured at 400 nm. The results were normalized to the permeability to arabinose. The sugars used were L-arabinose (M_r = 150), D-glucose and D-galactose (M_r = 180), *N*-acetyl-D-glucosamine (M_r = 221), sucrose and lactose (M_r = 342), raffinose (M_r = 504), and stachyose (M_r = 666). Envelope fraction from *P. aeruginosa* K799, as well as the PAO1 porin purified in dodecyl sulfate, produced results identical with those shown. The *broken line* shows, for comparison, results obtained with liposomes reconstituted with the OmpF porin from *E. coli* K12 (and crude envelope fraction from *E. coli* K12).

Third, the properties of the *P. aeruginosa* channel were not noticeably influenced by the presence of phospholipids or LPS from *P. aeruginosa*. As shown in Fig. 4, the effect of solute size remained essentially identical when one-tenth of the egg phosphatidylcholine was replaced with crude phospholipid mixture from *P. aeruginosa* PAO1 or when 12 nmol of *P. aeruginosa* LPS were added together with *P. aeruginosa* phospholipids. The absolute values of the rates of change in optical density were essentially unaltered by the addition of *P. aeruginosa* phospholipids, but addition of LPS decreased them by about 40%. However, this observation does not necessarily mean the alteration of pore-forming efficiency by LPS, as incorporation of large amounts of LPS into liposomes is known to change drastically the relationship between the volume and optical density of liposomes,[2] in part by the light scattering caused by LPS molecules themselves and also by changing the average number of lamellae and the average interlamellar distance presumably through the electrostatic repulsion between LPS molecules.[3] In addition, both the apparent swelling rate and the effect of the solute size were unchanged even when *P. aeruginosa* lipids were used exclusively for the preparation of liposomes (not shown).

Finally, we emphasize that the permeability of the liposomes was proportional to the amount of porin added (see Fig. 3 and especially the *inset*). This suggests that in order to produce channels, it is probably unnecessary to assemble larger structures from the incorporated porin units because concave upward curves would be expected under such conditions.

Quaternary Structure of Porin

Gel Filtration—P. aeruginosa porin appeared to become eluted as large aggregates from a Sephacryl S-200 column if gel filtration was done in cholate. Thus, the position of elution was much earlier than that of bovine serum albumin (66,000 daltons) (Fig. 5*a*). However, the porin peak was extremely broad, suggesting the presence of a population of heterogeneous aggregates, but not revealing the presence of any distinct oligomeric form.

In contrast, the *P. aeruginosa* porin was eluted as a sharp symmetrical peak in SDS (Fig. 5*b*). Because the protein was functionally active, it was expected to be in a more or less globular conformation. Thus, proteins that retained their globular shape in SDS, or not denatured by SDS, were needed as molecular weight standards. *E. coli* OmpF porin in its trimeric form (7) and *E. coli* OmpA protein (22, 23) were used for this purpose. Clearly, the *P. aeruginosa* porin was eluted at the position of the OmpA protein monomer, *i.e.* at the position of a 35,000-dalton (24) protein, significantly behind the position expected for trimers of proteins of this size range. We also

[2] H. Nikaido, unpublished results.
[3] T. Nakae and H. Nikaido, unpublished results.

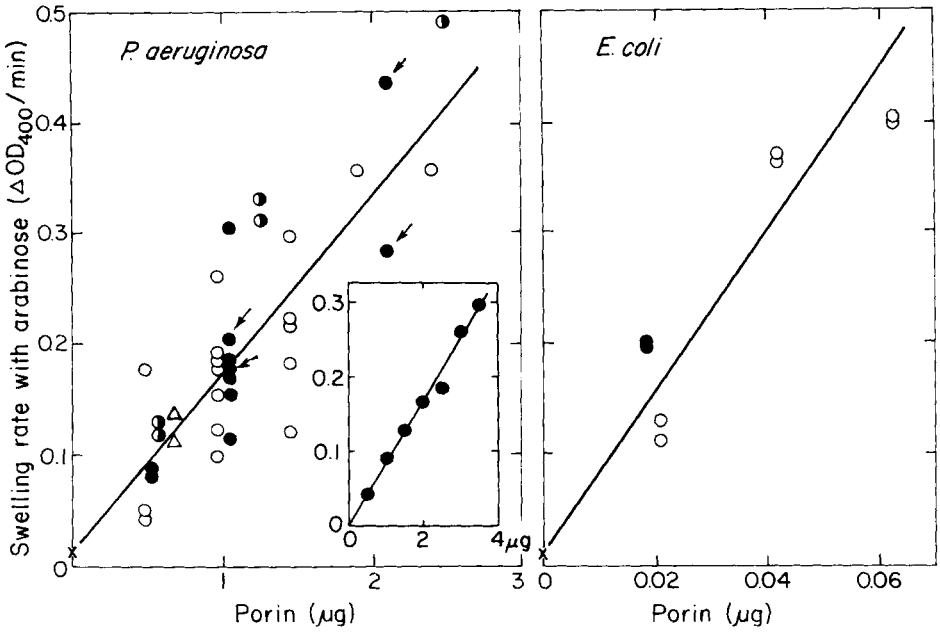

FIG. 3. **Swelling rates of liposomes reconstituted with *E. coli* OmpF porin or *P. aeruginosa* porin.** Specified amounts of porin (or membranes containing these amounts of porin on the basis of the scanning of Coomassie blue-stained SDS-polyacrylamide gels) were reconstituted with 2.25 μmol of egg phosphatidylcholine and 0.1 μmol of dicetylphosphate, and the permeability of the liposome membrane to L-arabinose was measured by the rate of swelling of liposomes upon dilution into isotonic L-arabinose solution. *Left,* porin purified in cholate (○), porin purified in dodecyl sulfate (◑), whole envelope (●), and outer membrane (△). *Arrows* show data points obtained with strain K799; other data were obtained with PAO1. The *inset* shows results obtained with a preparation of LDS-purified porin. Each *point* is an average of four to seven data points, and the standard error was, on the average, about 20%. The absolute values of swelling rates were lower in this experiment because dextran T-40 (Pharmacia) was used instead of dextran T-20 used in other experiments. *Right,* OmpF porin trimers (○) and crude envelope from *E. coli* K12 (●).

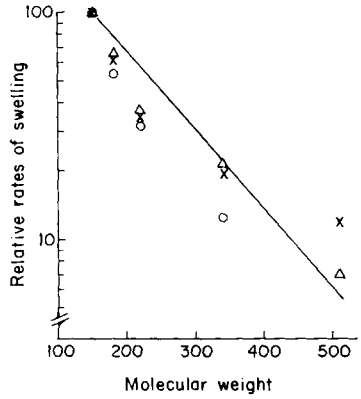

FIG. 4. **Effect of addition of *P. aeruginosa* phospholipids or *P. aeruginosa* LPS to reconstitution mixture on the permeability of porin-containing liposomes.** *P. aeruginosa* porin (10 μg) was reconstituted with 2.45 μmol of egg phosphatidylcholine (○), that plus 0.2 μmol of *P. aeruginosa* phospholipids (△), or these two phospholipid preparations plus 12 nmol of *P. aeruginosa* LPS (×), and the swelling rates of liposomes in 40 mM solutions of various sugars were measured. The rates were normalized to that in L-arabinose. Data points at M_r = 180 and 342 are averages of rates in D-glucose and D-galactose and those in sucrose and maltose, respectively. Data points at M_r = 221 and 505 were obtained by using *N*-acetyl-D-glucosamine and raffinose, respectively.

note that Mizuno and Kageyama (11) noted in their earlier study that outer membrane proteins were eluted from an SDS-equilibrated gel filtration column in the order of their monomer molecular weights, although comparisons with standards were not carried out.

Cross-linking Studies—Because the presence of functionally active, monomeric porin, suggested by the results described above, was quite unexpected, we tried to confirm these results by the use of covalent cross-linking reagents. When a peptidoglycan preparation with associated proteins such as porin and protein H was treated with dimethylsuberimidate, the proteins were apparently cross-linked into huge aggregates that failed to enter the separating gel (data not shown). Although this experiment showed that dimethylsuberimidate was an excellent cross-linking agent for *P. aeruginosa* porin, the results did not shed any light on the potential quaternary structure of this protein. When the porin purified in LDS was treated with dimethylsuberimidate, the reagent obviously reacted with the protein because the band of the porin seen in SDS-polyacrylamide gel analysis became much fuzzier. However, there was no formation of higher oligomers (Fig. 6). In contrast, *E. coli* OmpC porin treated under the same conditions was converted largely to what appeared to be trimers and dimers, confirming the trimeric structure of this protein.

Analytical Centrifugation Studies—For the purpose of determining the size of the active porin protein more precisely, analytical centrifugation studies were performed with the help of Ying Yang and Dr. H. K. Schachman of the Department of Molecular Biology. Porins were prepared by gel filtration in SDS as described under "Materials and Methods" and were equilibrated by an extensive dialysis against 0.4% SDS, 10 mM Tris-Cl, pH 8.0, 0.1 M NaCl. A portion of this preparation was denatured by precipitating with 10% (final concentration) trichloroacetic acid at room temperature and was dissolved in the dialysis buffer after two washes with ethyl ether. SDS-polyacrylamide gel analysis of these preparations showed that the "native" preparation did not contain any visible traces of the "denatured porin" (or F*) band corresponding to the apparent molecular weight of 40,000, whereas the "denatured" preparation consisted entirely of the "40,000-dalton" band.

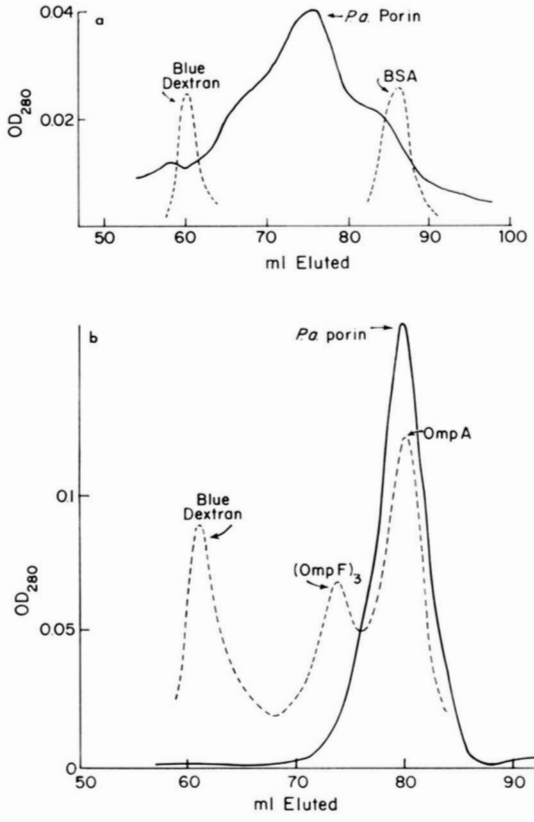

FIG. 5. **Elution profiles of *P. aeruginosa* porin.** *a*, gel filtration of *P. aeruginosa* porin purified in cholate. The sample was applied to a column (1.5 × 90 cm) of Sephacryl S-200 equilibrated with 1% Na-cholate, 1 M NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA and was eluted with the same buffer. The inclusion volume of this column, determined with ATP, was 156 ml. In spite of this broad and complex elution profile, fractions eluted at 65 through 87 ml all showed the presence of a single band of porin on SDS-polyacrylamide electrophoresis. Elution pattern of blue dextran and bovine serum albumin (*BSA*) in a separate run is shown by a *broken line*. *b*, gel filtration was performed on the same column, but equilibrated and eluted with 1% SDS, 0.4 M NaCl, 10 mM Tris-Cl buffer, pH 8.0. The absorbance of the effluent was continuously recorded by using an LKB Uvicord S monitor. The elution profile of a control mixture containing blue dextran, *E. coli* OmpF porin trimers, and *E. coli* OmpA protein is shown by a *broken line*, with some base-line displacement for clarity. OD_{280}, A_{280}.

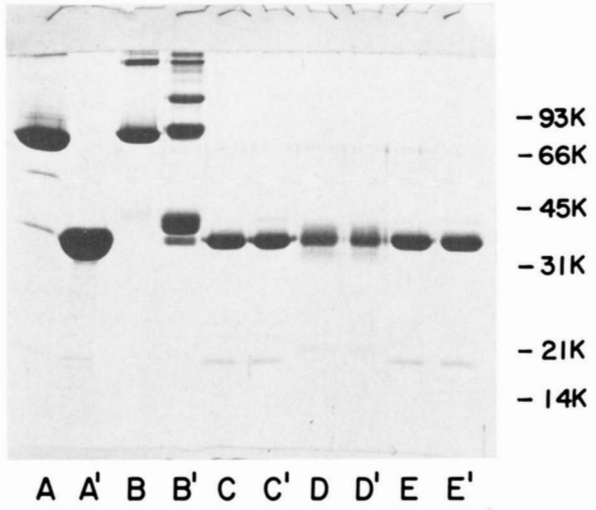

FIG. 6. **Attempted cross-linking of *P. aeruginosa* porin purified in dodecyl sulfate.** *P. aeruginosa* porin and *E. coli* OmpF porin (both in SDS) were treated with dimethylsuberimidate as described under "Materials and Methods." The products were applied to SDS-polyacrylamide electrophoresis without (*samples A–E*) or after (*samples A'–E'*) heating at 100 °C for 2 min. *A*, untreated *E. coli* OmpF porin; *B*, cross-linked OmpF porin; *C*, *P. aeruginosa* porin treated in the same way as *D*, but omitting dimethyl suberimidate; *D*, "cross-linked" *P. aeruginosa* porin; and *E*, untreated *P. aeruginosa* porin. *93K*, 93,000 daltons, for example.

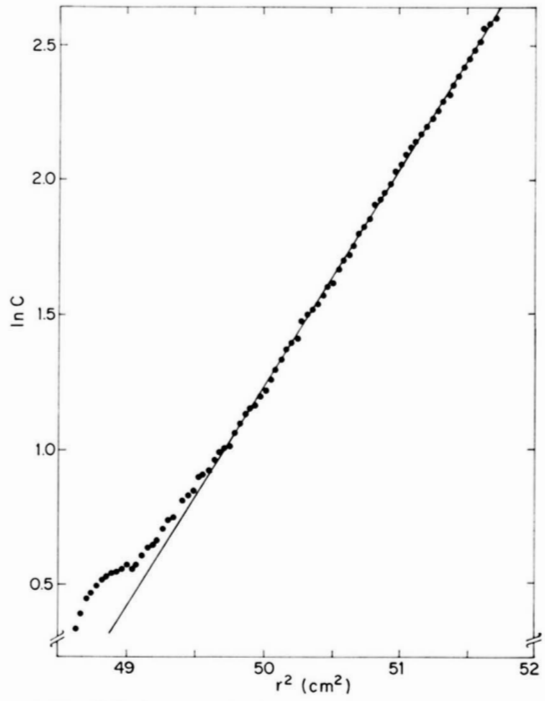

FIG. 7. **Equilibrium centrifugation of *P. aeruginosa* porin in SDS.** *P. aeruginosa* porin purified by gel filtration in SDS was equilibrated with 0.4% SDS, 0.1 M NaCl, 10 mM Tris-Cl buffer, pH 8.0, by extensive dialysis. The solution (0.7 mg of protein/ml) was centrifuged at 20 °C at 17,000 rpm in a Beckman analytical ultracentrifuge, and the concentration of the porin was determined by scanning at 280 nm. The figure shows the plot of the natural logarithm of optical density (*ln C*) *versus* the square of distance from the axis of rotation (r^2).

When these preparations were examined by velocity centrifugation at 60,000 rpm in a Spinco analytical centrifuge, photometric scanning at 280 nm revealed smooth meniscus without any sign of gross heterogeneity. The $s_{20,w}$ values were 2.89 ± 0.02 and 2.61 ± 0.04 for native and denatured porins. These values strongly suggested that the porins existed as monomers in the native state in SDS because of the following reasons. (i) Globular proteins of sizes comparable to the monomer of *P. aeruginosa* porin, *i.e.* 30,000–40,000 daltons, are known to show $s_{20,w}$ values of 2.8–3.8 (25). Thus, if the porin retains its more or less globular shape in SDS, then the observed $s_{20,w}$ value is what is expected for its monomer. This can be contrasted with the $s_{20,w}$ values of *E. coli* porin trimers, reported to be 6.8 (7) and 7.4 (18). (ii) With the *E. coli* porin trimer, denaturation results in its dissociation into monomeric units. Thus, the $s_{20,w}$ value decreases by a factor of 2 or more upon denaturation (7). In contrast, with *P. aeruginosa* porin, there was only a 10% decrease, presumably caused by the conversion into a more extended conformation.

We further sought to confirm the preliminary conclusion above by carrying out a sedimentation equilibrium run overnight at 17,000 rpm. The results (Fig. 7) were substituted into the equation of Tanford *et al.* (26), *i.e.*

$$M_r = \frac{d \ln C}{d(r^2)} \cdot \frac{2RT}{\omega^2} \cdot \frac{1}{[(1 - \bar{v}\rho) + \delta_D(1 - \bar{v}_D\rho)]}$$

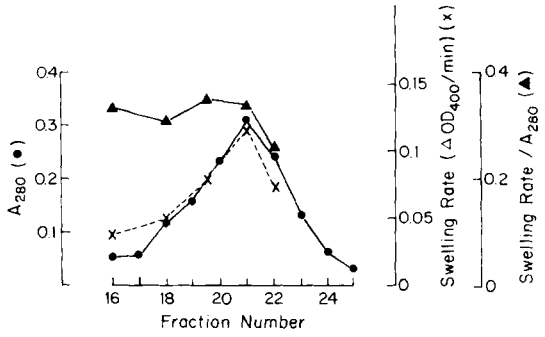

FIG. 8. **Pore-forming activity of *P. aeruginosa* porin across the "peak" of elution from a Sephacryl S-200 column.** *P. aeruginosa* porin (about 2 mg), purified in dodecyl sulfate, was applied to a column (1.5 × 90 cm) of Sephacryl S-200 equilibrated with 1% SDS, 0.4 M NaCl, 10 mM Tris-Cl, pH 8.0, and eluted with the same buffer at the flow rate of 3 ml/h. Each of the first 15 fractions contained 5 ml, and each of fractions 16–25 contained 1.2 ml. Fractions were dialyzed briefly against 0.1% SDS, 5 mM Tris-Cl, pH 8.0, 1 mM NaN_3 to reduce the content of NaCl, and 5 μl of each fraction were reconstituted with 2.25 μmol of egg phosphatidylcholine and 0.1 μmol of dicetylphosphate, and the swelling rates of these liposomes were measured in an isotonic solution of L-arabinose. SDS-polyacrylamide gel analysis of dialyzed fractions showed that a fraction of the porin has been converted into a denatured (or F*) form, presumably as a result of dialysis against the low ionic strength buffer. However, the fraction of the total porin in the denatured form was about the same (20–30%) in all of the fractions. The points between fractions 19 and 20 show the results of the assay with a 1:1 mixture of these two fractions. ΔOD_{400}, ΔA_{400}.

where C, r, ω, $\bar{v}$, ρ, δ_D, $\bar{v}_D$, R, and T denote solute concentration, distance from the center of the rotor, angular velocity, partial specific volume of the protein, the density of the solvent, the amount of detergent bound to unit mass of the protein, partial specific volume of the detergent, gas constant, and temperature (in Kelvin), respectively. By using the value of $\bar{v} = 0.72$ (calculated from amino acid composition (11) according to Ref. 27), $\rho = 1.008$ (gravimetrically determined), $\delta_D = 0.7$ g/g of porin (determined by gel filtration as described under "Materials and Methods"), and $\bar{v}_D = 0.87$ cm^3/g (26), the molecular weight of the native *P. aeruginosa* porin was calculated as 35,000. Since this value was obtained without introducing any arbitrary assumptions on the shape of the molecule, it is clear that the functionally active *P. aeruginosa* porin exists as a monomer in SDS.

Channel-forming Activity of Individual Fractions from Gel Filtration—Since the *P. aeruginosa* porin produced a very low degree of permeability upon reconstitution and since the majority of the population of porin in LDS were monomers, we considered the possibility that the monomers were devoid of channel-forming activity, and only the very rare oligomers could produce the channel, thus accounting for the low permeability. If this hypothesis were correct and if the oligomeric structure were stable, then the pore-forming activity/unit weight of protein should be very high at the leading edge of the porin peak eluted from the gel filtration column and should decrease to a very low value in the main portion of the peak. However, such fluctuations in the specific activity were not observed (Fig. 8).

DISCUSSION

In this study, we have shown that functionally active porin preparations could be prepared from *P. aeruginosa* outer membrane or envelope fractions in the presence of LDS or SDS. This observation is consistent with the results of Mizuno and Kageyama (11), who found that *P. aeruginosa* porin in SDS was rich in β-sheet structure, just as in the native, functionally active porins of *E. coli* (7, 23) and *S. typhimurium* (8). We confirmed the observation of Mizuno and Kageyama (11) that very prolonged heating at 100 °C, up to 1 h or more, is needed to denature the *P. aeruginosa* porin completely; thus, it appears that *P. aeruginosa* porin is even more heat-stable than the porins of enteric bacteria. We found, however, that *P. aeruginosa* porin was easily denatured by exposure to low ionic strength medium in the presence of SDS, and this property may explain the previous failure to obtain an active *P. aeruginosa* porin preparation in SDS.

The measurement of solute diffusion rates by the liposome-swelling technique produced several significant pieces of information on the properties of *P. aeruginosa* porin channel. The size of the channel appeared to be larger than that in *E. coli*, and the slope of the permeability *versus* the hydrated radius of the solute plot (Fig. 2, also see Ref. 13) appeared to fit best with what was predicted by the Renkin equation (21) for a pore of about 1.0-nm radius. This estimate is in good agreement with the value of 1.1 nm calculated by Benz and Hancock (6) from the single channel conductivity experiments, although we feel that the use of bulk conductivity in these calculations is not completely justified (28). This is 1.72 times larger than the estimated radius of the *E. coli* pore, 0.58 nm (13). Since the weight of the globular molecules should increase in proportion to the cube of its radius and since the "exclusion limit" for the *E. coli* channel was about 600 daltons (29), the expected exclusion limit for the *P. aeruginosa* channel is 600 × $(1.0/0.58)^3$ = 3072 daltons; this value is close to the previous estimate of 3000–9000 daltons, determined on the basis of the efflux of labeled heterodisperse dextran molecules (5).

The most striking characteristic of *P. aeruginosa* outer membrane is its low permeability toward a wide variety of solutes. We have shown that the permeability coefficients of *P. aeruginosa* outer membrane were indeed 10–100-fold lower than those of *E. coli* outer membrane (3). It is gratifying to note that this difference in permeability, or the rate of penetration of solutes when driven by a unit force, was also found with the purified porin (Fig. 3); this low permeability in the reconstituted system is not likely to be due to an artifactual inactivation of porins during purification, as porins purified in different detergents as well as fragments of crude outer membrane showed the same low permeability (Fig. 3). A similarly low permeability was also noted in a study of *P. aeruginosa* porin reconstituted into black lipid films (6).

Thus, the low permeability of *P. aeruginosa* outer membrane can be largely explained by the intrinsic low permeability of its porin channels, but the molecular mechanism underlying this latter observation is still not clear. *A priori*, one can consider two possibilities. Either each channel is constructed to produce a great deal of resistance to the penetration of solutes, or only a few among the population of channels are in the "open" configuration. Benz and Hancock (6) showed, by using the black lipid film technique, that the permeability of a single *P. aeruginosa* porin channel was rather large; this result strongly supports the latter interpretation. Teleonomic considerations suggest that the closed porin channels may become open whenever physiological conditions demand it. One such condition would be nutrient starvation. In our previous chemostat experiments (3), limiting availability of nutrients to a moderate degree did not appear to increase the permeability of the outer membrane drastically. However, severe nutrient limitation conditions have not been tried, and more work is needed in this area.

One unexpected observation was the finding that monomers of *P. aeruginosa* porin were fully active in forming channels in the reconstituted vesicles (Figs. 3 and 5–8). This was

unexpected because porins previously studied, *e.g.* those of *E. coli* (7), *S. typhimurium* (8), *Neisseria gonorrhoeae* (30, 31), and *Brucella abortus* (32),[4] were always found to exist in oligomeric (usually trimeric) structures and their monomers were totally incapable of forming transmembrane pores in reconstitution assays (7, 8) and because *a priori* it was difficult to imagine that a 36,000-dalton protein could span the thickness of the outer membrane and could contain a large stable channel in the center. It is still possible that the monomers aggregate together in the phospholipid bilayers and that only the aggregated forms contain the channel. If so, the permeability of liposomes is likely to show an upward concave dependence on the amount of porins added. The dependence, however, was linear (Fig. 3) and, thus, our results tend to favor the possibility of each monomer producing an independent channel. Very recently, it was found that a stable channel was produced even by a 20,000-dalton fragment of colicin E1 (33),[5] and in view of such a finding, it may not be surprising that a 35,000-dalton protein could produce a large stable channel.

We emphasize, however, that the activity of the monomeric porin does not necessarily indicate that the *P. aeruginosa* porin molecules exist as monomers in the outer membrane. Further work is needed in this area; most recently, on the basis of cross-linking studies, it has been claimed that *P. aeruginosa* porin existed as trimers in the membrane.[6]

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