Porin is a trimeric membrane protein that functions as a diffusion pore in the outer membrane of *Escherichia coli*. We report the existence and purification of porin heterotrimers between the *ompC*, *ompF*, and *phoE* porin gene products. Separation was achieved using a high resolution anion exchange column. The amount of each heterotrimer species present depended on the level of expression of the subunits and was consistent with random mixing of trimer subunits. A strong effect of bacterial lipopolysaccharide on the chromatography of porin was also detected. These results imply that assembly of porin trimers occurs between subunits synthesized on different polysomes and that subunit contacts between the porin subunits occur in conserved regions of the primary sequence.

*Escherichia coli* K12 produces two species of porin, products of the *ompC* and *ompF* genes, in ordinary culture media and a third porin, a product of the *phoE* gene, under conditions of phosphate starvation. The expression of the *ompC* and *ompF* genes is regulated in response to the osmolality of the medium. Under conditions of high osmolality *ompC* is predominantly expressed, and under conditions of low osmolality *ompF* is predominantly expressed. The *phoE* gene can be expressed by either growth under phosphate limitation or in nutrient broth (NB, 8 g of Difco nutrient broth/liter) with additions of NaCl as indicated. Homotrimers of OmpF, OmpC, and PhoE were purified from the mutant strains JF701 (ompC264), JF703 (ompF254), and JF694 (ompC264 ompF254 phoTS) as described by Nikaido et al. (1985).

For coexpression of PhoE with either OmpF or OmpC, strains JF701 and JF703 were grown in 70% strength MOPS medium (Neidhardt et al., 1974) with 1 mM β-glycerophosphate as sole phosphate source, 0.4% glucose, and essential nutrients as required. HN498, a PhoE constitutive strain, was constructed by transduction of DK8 to unc (growth on succinate as a carbon source) with a P1 vir lysate grown on JF694 (unc pheTS) and screening for constitutive alkaline phosphatase activity (Argast and Boos, 1980; Tommassen and Lugtenberg, 1980). HN499 was constructed by transduction of HN498 to kanamycin resistance with a P1 vir lysate grown on HN433 (ompF:Trs5). For the preparation of porin extracts, HN498 was grown in LB.

**Preparation of Porin Extracts**—Cells were harvested from exponentially growing cultures at an OD₅₇₀ = 1 (Hitachi 100-40 spectrophotometer) and washed once in 30 mM Tris/Cl, pH 7.5. The cell pellet (1–1.5 g, wet weight) was resuspended in 40 ml of cold 30 mM Tris/Cl, 1 mM MgCl₂, pH 7.5, containing 0.1 mg/ml each of pancreatic DNase I and RNase B (Sigma). The cell suspension was passed through a French pressure cell once at 100,000 × g for 1 h, the supernatant discarded, and the pellet gently rinsed with distilled water. Porin was extracted from the pellet by resuspension in 1.1 ml of a solution containing 1% SDS, 0.5 M NaCl, 30 mM Tris/Cl, and 10 mM EDTA, pH 7.5. After brief sonication (2 min) in a bath sonicator and a 30-min incubation at 37 °C, the turbid suspension was centrifuged for 15 min at 100,000 × g and the supernatant liquid kept as the porin extract.

**Gel Filtration Chromatography**—To purify porin, 1 ml of the porin extract was applied to a 120-ml bed volume Sephacryl S-200 column equilibrated with 0.1% SDS, 0.4 M LiCl, 50 mM Tris/Cl, 0.1 mM EDTA, 3 mM NaN₃, pH 7.5, at 20 °C. The column was eluted with the same buffer, and peak fractions containing porin were pooled and dialyzed at room temperature overnight against 4 liters of 10 mM Tris/Cl, 3 mM NaN₃, pH 7.5. After dialysis, the zwitterionic detergent Zwittergent 3-14 (Boehringer Mannheim) was added to 0.1% (w/v).
Porin was stable in this buffer but showed an increase in peak widths on the Mono-Q column after storage for several months at room temperature.

**Mono-Q Chromatography.**—For analytical separations, 0.5 ml of porin purified as above was applied to a Pharmacia Mono-Q HR anion exchange column, equilibrated with 0.1% Zwittergent 3-14, 15 mM Tris/NaOH, 0.1 mM EDTA, pH 8.0, and the column eluted with a concave salt gradient of 0-300 mM Na₂SO₄ in 40 ml at a flow rate of 0.5 ml/min. Detection at 280 nm and quantitation of the eluted peaks were performed using a Perkin-Elmer LC-80 flow-through UV detector and a model 15 data analysis station.

**SDS-PAGE—SDS-urea gels** were used to separate OmpC from either PhoE or OmpF. Solid urea (6 M) was added to the separating gel of our standard SDS-PAGE system (Lugtenberg et al., 1975) with no other modifications. For separation of PhoE and OmpF, Pharmacia 10-15% SDS-Phastgels were used.

### RESULTS

In the initial study, porin trimers purified from strains producing only one type of porin subunit were subjected to chromatography on a Mono-Q anion exchange column (Table II). The elution of E. coli K12 OmpF earlier than K12 OmpC was consistent with the fact that the net negative charge/subunit at neutral pH is 10 and 14, respectively (Mizuno et al., 1983). However, the number of formal charges alone could not completely explain the elution behavior of the trimers because PhoE, which has even fewer net negative charges (−9) than OmpF (Mizuno et al., 1983), was eluted later than OmpF. In addition, OmpF and OmpC monomers (obtained by heat denaturation) were not resolved on the Mono-Q column. Preliminary experiments with the porins of Salmonella typhimurium revealed that these proteins share retention times similar to that of E. coli PhoE (data not shown). Also of interest is that both native and denatured E. coli B/r OmpF trimers showed different retention times on E. coli K12 OmpF, reinforcing suggestions that significant differences exist between the OmpF proteins of these two strains (Rosenbusch, 1974).

The large differences in retention times between OmpF and OmpC encouraged us to search for heterotrimers between these proteins. In order to do this, porin was purified by a simple SDS/NaCl extraction procedure from cultures of JF568 (ompC* ompF*) grown under conditions that alter the relative expression of ompF and ompC. As shown in Fig. 1B, the relative amounts of OmpF and OmpC varied from approximately 2:1 to 1:2 as the salt concentration in the growth media was increased. When the purified porins were subjected to analytical Mono-Q chromatography, four peaks were observed (Fig. 1A). The peak fractions from a preparative separation were analyzed by SDS-urea-PAGE (Ichihara and Mizushima, 1979) for subunit composition, and it was found that the first peak was composed of only OmpF subunits, the second peak of a 2:1 mixture of OmpF and OmpC subunits, the third peak of a 1:2 mixture of OmpF and OmpC subunits, and the fourth peak of only OmpC subunits (Fig. 2A). Rechromatography on Mono-Q of material from each peak yielded only single peaks with retention times identical to the original peaks (Fig. 3).

As the relative expression of ompF and ompC changed, the relative peak heights for the four trimmer species also changed (Fig. 1A). Under conditions producing nearly equal expression of ompF and ompC (growth in NB + 0.5% NaCl), the ratio of the areas of the four peaks was 1:2:2:2:5:1. This suggests that nearly random subunit mixing occurs during the process of trimer association. As a control for an artifact of extraction or purification, two cultures of JF703 (ompF+) and JF701 (ompC−) were mixed before French pressing and purification of porin trimers. This mixed cell suspension yielded only single peaks with retention times identical to the original peaks (Fig. 3).

### Table II

| Protein* | Retention time | Source |
|----------|----------------|--------|
|**K12 OmpF** |                |        |
| Native    | 12.0           | Nikaido et al., 1983 |
| Denatured | 10.7           | Nikaido et al., 1983 |
|**K12 OmpC** |                |        |
| Native    | 15.0           | Nikaido et al., 1983 |
| Denatured | 10.6           | Hall and Silhavy, 1981 |
|**B/r OmpF** |                |        |
| Native    | 15.0           | Klionsky et al., 1994 |
| Denatured | 13.3           | This study |
|**K12 LamB** |                |        |
| Native    | 15.0           | This study |
| Denatured | 11.8           | This study |
|**K12 PhoE** |                |        |
| Native    | 13.4           | This study |
| Denatured | 10.4           | This study |

* Outer membrane proteins were purified from either E. coli K12 or B/r as indicated. Proteins were either applied as native trimers or boiled for 15 min in Zwittergent buffer to denature them to mono-meric subunits.

* Column conditions were as described under "Materials and Methods" except that the 0-300 mM gradient was run in 20 min at 1 ml/min.
Porin Heterotrimers from E. coli Outer Membrane

FIG. 1. Analysis of purified OmpF/OmpC porin in relation to different growth conditions. A. Mono-Q chromatograms of porin purified from JF568 (ompF<sup>+</sup>, ompC<sup>+</sup>) grown in different media as indicated. The bottom tracing is a chromatogram of porin purified from a mixture of JF703 (ompF<sup>-</sup>) and JF701 (ompC<sup>-</sup>) cells grown in LB. The four main peaks in the chromatograms correspond, from left to right, to OmpF homotrimers, OmpF<sup>/</sup>OmpC<sup>+</sup> heterotrimers, OmpF<sup>+</sup>/OmpC<sup>-</sup> heterotrimers, and OmpC homotrimers. B. Densitometric scans of SDS-urea-PAGE analysis of the purified porins showing the relative amounts of OmpC and OmpF subunits present. The preparations shown in B are the same materials used in the analyses in A. In several preparations, a small amount of contaminating LamB and OmpA was present that did not interfere with subsequent analysis. No other bands were visible in the gels.

peaks (Fig. 4). SDS-PAGE analysis of the eluted peaks showed that the first peak consisted of only OmpF, the second peak consisted of OmpF and PhoE in an approximate 2:1 ratio, the third peak consisted of OmpF and PhoE in a 1:2 ratio, and the fourth peak consisted of only PhoE (Fig. 2B). Surprisingly, porin from JF703 (ompF<sup>-</sup>) was resolved into only three peaks on the Mono-Q column (Fig. 4). SDS-PAGE analysis of these peaks established that the third peak (at 27 min) consisted only of OmpC but was unable to identify clearly the composition of the first two peaks (data not shown).

In order to produce more PhoE protein, a PhoE constitutive strain was constructed. This strain, HN499, produced large amounts of PhoE and smaller amounts of OmpC. When applied to the Mono-Q column, porin from HN499 was also resolved into only three peaks (Fig. 4). SDS-urea-PAGE analysis of this material allowed identification of the composition of the first two eluted peaks (Fig. 2C). The first peak did not show a constant subunit composition but represented material from a PhoE homotrimer peak and a PhoE/OmpC mixed trimmer peak that was incompletely resolved. The leading edge of the first peak (Fig. 2C, fractions 1 and 2) consisted of essentially only PhoE, while the trailing edge (fractions 6–8) consisted of PhoE and OmpC in a 2:1 ratio. The second peak (fractions 10–15) consisted of PhoE and OmpC in a 1:2 ratio. The third peak (fractions 22–28) consisted of only OmpC with contamination by LamB that coelutes with OmpC homotrimers. No heterotrimers were detected between LamB and any of the porins.

When porin extracts were applied to the Mono-Q column before gel filtration chromatography, all of the porin proteins eluted late (at around 30 min on a 40-min gradient). A mixing experiment with gel filtration-purified porin and a dialyzed porin extract showed that the porin extract contained a nondialyzable constituent that interfered with Mono-Q chromatography of porin even at high dilutions (data not shown). It appeared that this constituent was bacterial lipopolysaccharide (LPS). When near stoichiometric amounts of LPS were added to purified PhoE porin, the protein showed a marked retardation in the elution time (Fig. 5). At higher LPS concentrations, the chromatogram was essentially identical to that of unpurified porin in SDS/NaCl extracts.

DISCUSSION

We have taken advantage of the high resolving power of a fast protein liquid chromatography ion exchange column to...
Porin Heterotrimers from E. coli Outer Membrane

The observed ratios of the four trimer species between OmpF and OmpC support a model of essentially random mixing of the subunits before trimerization. If the fraction of OmpC to total porin (OmpC + OmpF) is represented as \( \beta \), then the expected ratios of the four trimer species based on a random mixing model are 
\[
(\beta^3, 3\cdot\beta^2(1-\beta), 3\cdot\beta(1-\beta)^2, (1-\beta)^3)
\]
We have used two methods to analyze our data. The first is a simple comparison of relative peak heights using a computer simulation based on the above formula. This simulation shows a good fit for the first three growth conditions shown in Fig. 1: NB, NB 0.25% NaCl, and NB 0.5% NaCl. The fit is slightly less good for the higher salt conditions: NB 1% NaCl and LB. The Mono-Q chromatograms show more OmpC homotrimers than would be expected on the basis of the amount of OmpC detected in the gel scans. One possible explanation is coelution of a small amount of contaminating LamB with the OmpC homotrimers. Another possibility is that an increase in OmpC expression occurred as the cells entered the late exponential growth phase.

We have also analyzed the ratio of heterotrimers to homotrimers based on the following formula,
\[
\alpha = \frac{(\text{area peak 2} - \text{area peak 3})}{(\text{area peak 1} - \text{area peak 4})^2}
\]
where \( \alpha \) is a measure of the tendency toward random mixing. For completely random mixing of subunits, \( \alpha = 3 \). When there is no mixing, \( \alpha = 0 \). Analysis of the chromatograms shown in Fig. 1 gives values for \( \alpha \) between 1.5 and 2.0. Analysis of a preparative chromatogram of porin from cells grown in NB + 0.5% NaCl gives \( \alpha = 2.35 \). These values for \( \alpha \) imply that there is a small tendency for the porin subunits to self-associate preferentially into homotrimers. It is important to note, however, 1) that the area measurements are subject to large errors and the intermediate heterotrimer peaks may be systematically underestimated; and 2) that any variation in \( \beta \) during the growth of the cells will tend to decrease \( \alpha \). Therefore, the observed values for \( \alpha \) correspond to the lower limits. The effect of variations in \( \beta \) is especially clear in the experiments with PhoE induced with \( \beta \)-glycerophosphate (Fig. 4). Porin from these cultures shows a low ratio of heterotrimers to homotrimers probably due to uneven expression of PhoE. For OmpF/PhoE, \( \alpha \) equals only 0.9. In porin from the strain constitutive for PhoE (HN499), however, where the expression of PhoE is expected to be more even, the ratio of heterotrimers to homotrimers appears to be much higher.

In light of the existence of heterotrimers between OmpC and OmpF, it is not surprising that PhoE also forms mixed trimers. The three proteins show a similar high degree of relatedness suggesting a common ancestral gene. Comparison of OmpC with PhoE shows 76% identity or conservative substitutions and with OmpF 73% identity or conservative substitutions (Inokuchi et al., 1982; Mizuno et al., 1983;...
Overbeeke et al., 1983). Two other related porin proteins, NmpC and Lc, also exist in certain E. coli strains (Blasband et al., 1986). Based on the high homology of these proteins with OmpF, OmpC, and PhoE, they would also be expected to form mixed trimers. The existence of mixed trimers between porins suggests that the subunit contacts are highly conserved. Other studies have also suggested that the transmembrane domains of the porin genes are conserved while sequence divergence has occurred to the greatest extent in portions exposed outside of the membrane (Tommassen, 1988).

Examination of Fig. 1 shows that under most growth conditions the majority of porin trimers are heterotrimers. We have not examined porin from a strain producing PhoE, OmpC, and OmpF, but theory would predict the existence of 10 different trimer species including a mixed trimer of PhoE, OmpF, and OmpC in a 1:1:1 ratio.

The mixing of subunits implies that trimerization of porin monomers occurs between subunits made on different polymers. This strongly suggests that the assembly of porin trimers is a distinct process, uncoupled from protein translation. Assembly is nonetheless a rapid process with a half-life of only 20 s in growing cells as determined by pulse-chase experiments (Reid et al., 1988). Mixing of monomers made on different polymers has also been detected in the case of the LamB protein. The analysis by Marchal and Hofnung (1983) of negative dominant phage-resistant mutations in LamB concluded that phage-resistant heterotrimers of LamB exist in cells carrying a phage-resistant and phage-sensitive (wild-type) LamB gene.

Ichihara and Mizushima (1979) demonstrated that the porin subunits do not mix after incorporation into trimers. In their elegant experiment, they shifted cultures from OmpC- to OmpF-producing conditions (and vice versa) and showed that this reduced the quantity of cross-linked heterodimers relative to homodimers. In light of their result and the extreme stability of porin trimers, it seems highly unlikely that porin subunits can rearrange in the membrane after synthesis. Thus, as assumed in the discussion of \( \alpha \), the ratio of porin subunits at synthesis determines the final ratios of the four trimer species.

The effect of LPS on the chromatography of porin on the anion exchange Mono-Q column appears to reflect two processes. At low concentrations, LPS retards the elution of porin trimers, and the addition of as few as 1.5 LPS molecules/porin trimer noticeably decreases column resolution (Fig. 5); this observation suggests the presence of a specific LPS-porin interaction. On the other hand, at higher concentrations (~30 \( \mu \)g/ml), LPS not only retards the elution of porin trimers more strongly but also retards slightly the elution of denatured porin monomers and bovine serum albumin (data not shown). Chromatography of other proteins such as ovalbumin is not affected by similar amounts (data not shown). This second effect may reflect low affinity binding of LPS to hydrophobic segments of the proteins. There are several precedents in the literature for a specific interaction between porin and LPS. Yu and Mizushima (1977) reported that LPS stimulated porin binding to peptidoglycan, and Yamada and Mizushima (1980) noted a requirement for at least one LPS molecule/porin trimer for formation of two-dimensional crystalline arrays of porin. (Note, however, that other amphiphilic compounds could substitute for LPS.) Similarly, Schindler and Rosenbusch (1981) reported that LPS affects the conductance properties of porin in membrane bilayers. Both Farr et al. (1986) and Rocque et al. (1987) have described LPS binding to purified porin.

When porin is inserted into lipid bilayers, single channel conductance steps can be seen (Benz, 1985). These conductance steps are believed to be due to insertion of whole trimers in the membrane or to the coordinated opening of all three channels in the trimer. In contrast, Engel et al. (1985) have observed voltage-induced individual channel-closing events in B/r OmpF trimers. The significance of these findings is further confused in the wake of the recent report that the voltage-gating phenomenon observed in black lipid experiments is not physiologically relevant (Sen et al., 1988). The existence of purified preparations of heterotrimers should allow testing of these hypotheses. If individual pores can open and close, then recordings of mixed trimers should show two different conductance steps. Conversely, if the only event is insertion or opening of whole trimers, then the heterotrimers should show only a single conductance step size intermediate between those observed with OmpF and OmpC homo trimers.

We are currently examining the black lipid conductance properties of the porin heterotrimers.

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