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Rapid Isolation of OmpF Porin–LPS Complexes Suitable for Structure–Function Studies†

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ABSTRACT: A gentle and rapid isolation procedure is described yielding fractions containing better than 95% pure OmpF porin of *Escheria coli* B^E with different amounts of bound lipopolysaccharide (LPS). The procedure employs continuous free-flow electrophoresis (FFE) in the presence of detergent above its critical micelle concentration. Total yields of around 45% were typically obtained when porin-enriched membrane extracts were processed. By use of analytical ultracentrifugation a molecular mass of 114 000 and a sedimentation coefficient $s_{20,w}$ of 5.0 S were determined for porin trimers containing approximately 1 mol of tightly bound LPS. This porin readily formed 3D crystals suitable for high-resolution X-ray diffraction analysis. Three other porin–LPS isoforms isolated by FFE revealed molecular masses of 120 000, 124 000, and 151 000, suggesting that, in addition to the tightly bound LPS, 1, 2, and 8 mol of loosely bound LPS were present per mole of porin trimer. Each of the four different isoforms was suitable for reconstitution into highly ordered protein–lipid membrane arrays. The membrane crystals obtained with the 151-kDa isoform exhibited a unit cell polymorphism similar to that previously reported.

A major function of the outer membrane of Gram-negative bacteria is to serve as a permeability barrier. To this end, various pore-forming integral membrane proteins such as OmpC, OmpF (porin), LamB (maltoporin), and PhoE (phosphoporin) have been demonstrated to play an important role in the outer membrane of *Escherichia coli* (Lugtenberg & Van Alphen, 1983; Benz, 1985; Nikaido & Vaara, 1985). *E. coli* B^E porin has been the subject of extensive structural (Dorset et al., 1983; Garavito et al., 1983; Engel et al., 1985) and functional (Schindler & Rosenbusch, 1978, 1981) investigations. The functional porin unit is a trimer consisting of three 37.2-kDa polypeptides (Chen et al., 1982) forming three separate transmembrane channels at the outer surface of the cell that merge into a single channel at the periplasmic side of the outer membrane as revealed by three-dimensional (3D) electron microscopy of two-dimensional (2D) porin crystals (Engel et al., 1985). These hydrophilic water-filled pores with a physical diameter of approximately 1 nm facilitate diffusion of ions and hydrophilic solutes below a molecular mass of approximately 600 (Nikaido & Nakae, 1979). In addition to their size, the hydrophilicity (Nikaido et al., 1983) and the charge (Benz et al., 1979) of the solutes have been reported to affect the permeability coefficients of the pores. In contrast to phospho- (Korteland et al., 1982; Overbeeke & Lugtenberg, 1982) and maltoporin (Ferenci et al., 1980; Luckey & Nikaido, 1980), as yet no substrate-binding proteins located on the outer periphery of the porin channels have been identified. Furthermore, porin trimers are reported to exhibit at least two functional states—an open and a closed one—that are inducible upon application of a membrane potential

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(Schindler & Rosenbusch, 1978). This voltage dependence of the permeability of the porin channels allows the monitoring of channel activity by evaluating the voltage-gated conductance of reconstituted membranes (Schindler & Rosenbusch, 1981). This assay revealed that the presence of stoichiometric amounts of lipopolysaccharide (LPS) is required for voltage-gating of porin channels (Schindler & Rosenbusch, 1981). LPS is exclusively found in the outer membrane where it is associated with porin (Yamada & Mizushima, 1980; Strittmatter & Galanos, 1987). However, it is still a matter of discussion how critically the structure and function of porin depend on the presence of LPS. While several authors have reported that porin is functional only in the presence of LPS (Schindler & Rosenbusch, 1981; Lugtenberg & Van Alphen, 1983; Hitchcock & Morrison, 1984) with the lipid moiety of LPS being the essential component (Nakae et al., 1979), others have argued against a functional importance of LPS (Parr et al., 1986). Since the actual role of LPS still remains elusive, there is a need for a purification scheme avoiding any treatments that may result in an uncontrollable loss of LPS such as SDS extraction or EDTA/ethanol precipitation (Rosenbusch, 1974; Garavito & Rosenbusch, 1986; Parr et al., 1986; Worobec et al., 1988). Furthermore, a stepwise removal of porin-bound LPS would provide the basis for a systematic investigation of LPS-specific effects on porin structure and function.

Here we present a rapid and gentle OmpF porin purification procedure that is in addition capable of separating porin trimers according to the amount of loosely bound LPS, yielding different porin-LPS isoforms. The procedure is based on continuous free-flow electrophoresis (FFE) (Svensson & Brattsten, 1949; Grassmann, 1951; Bier, 1957; Barrolier et al., 1958; Hannig, 1961, 1964; Wiek, 1964; Wagner & Kessler, 1984) in the presence of 1% (v/v) octyl(polydisperse)oligooxyethylene (critical micelle concentration 0.23%) employing porin-enriched *E. coli* B^E membrane extracts as the starting material. All porin–LPS isoforms isolated by this procedure are suitable for reconstitution into large and highly ordered 2D protein–lipid arrays, whereas 3D crystals could only be grown with porin containing solely tightly bound LPS. Finally, we suggest that the use of FFE may have a general application for the gentle and effective purification of other membrane proteins.

Materials and Methods

Chemicals. DNase I (grade II) and RNase were purchased from Boehringer (Mannheim, FRG) and L-α-dimyristoylphosphatidylcholine (DMPC) and DL-dithiothreitol (DTT) from Sigma (St. Louis, MO). With the exception of bromophenol blue (Fisher, Fair Lawn, NJ), β-mercaptoethanol (Merck, Darmstadt, FRG), and SDS (Sigma), reagents for gel electrophoresis were obtained from Bio-Rad (Richmond, CA). Samples of the nonionic detergent octyl(polydisperse)oligooxyethylene (octyl-POE) (Rosenbusch et al., 1982) were a generous gift from Dr. J. P. Rosenbusch (Biocenter, Basel) from whom it can be obtained for specified experiments. The similar detergent octylpentaoxyethylene (cmc 4.3 mM) is commercially available (Bachem, Bubendorf, Switzerland). For any other detergents that may substitute for octyl-POE (cmc 6.6 mM) the reader is referred to a list given by Garavito and Rosenbusch (1986). All other reagents used were of analytical grade.

Cell Culture. E. coli B^E cells [BZB 3333; cf. Rosenbusch (1974) and Garavito and Rosenbusch (1986)], kindly provided by Dr. J. P. Rosenbusch (Biocenter, Basel), were grown in a 100-L fermenter as described previously (Garavito & Rosenbusch, 1986). After 3 h, typically 250 g of cells (wet weight) were harvested in a continuous-flow centrifuge and subsequently washed with 1 L of 50 mM sodium phosphate, 0.1 M NaCl, and 3 mM NaN_3, pH 7.6 (20 min at 10000*g*). Ready-for-use portions (85–90 g) of the washed cell paste were transferred into a deep-freezer and stored at −70 °C.

Crude Membrane Preparation and Solubilization of Porin. Unless otherwise stated, all incubation steps were carried out in a gyrotory water bath shaker at 37 °C and all centrifugations at 4 °C. Cells (85–90 g) were thawed in 250 mL of 50 mM sodium phosphate, pH 7.6, incubated for 10 min in the presence of 5 mg of DNase I, and centrifuged for 10 min at 8000*g*. Pellets were resuspended in 250 mL of sodium phosphate, pH 7.6, and homogenized before EDTA was added to a final concentration of 10 mM. After another low-speed centrifugation step (10 min at 8000*g*), the pellet was homogenized and incubated for 60 min in 60 mL of "breaking" buffer (50 mM sodium phosphate, 5% sucrose, and 3 mM NaN_3, pH 7.6) containing 6 mg of DNase I and 3 mg of RNase. The cells were then broken by double-passage through a French press at 1100 psi. Debris and unbroken cells were pelleted (10 min at 8000*g*). The supernatant was centrifuged for 80 min at 38800*g*, yielding a crude membrane pellet. Porin was extracted from crude membrane preparations by roughly following the porin-selective fractional extraction procedure described by Garavito and Rosenbusch (1986). Each extraction step consisted of a thorough resuspension of the membrane pellet in 100 mL of detergent-containing buffer followed by a 60-min incubation. After the incubation, the suspension was subjected to centrifugation (30 min at 142000*g*), yielding an extract and a pellet. Resuspension of the pellets was performed with a Teflon/glass homogenizer, using an extraction buffer (20 mM sodium phosphate, 2 mM DTT, and 3 mM NaN_3, pH 7.6) containing 0.5% octyl-POE for extraction steps 1–8 and 1% octyl-POE for extraction steps 9–12 (porin-enriched extracts).

Purification of Porin. Porin was purified from 1% octyl-POE porin-enriched extracts by employing preparative continuous free-flow zone electrophoresis (FFE) carried out in an Elphor VaP 21 (Bender & Hobein, München, FRG; separation chamber dimensions: 250 × 100 × 0.8 mm). All experiments were performed at 800 V/110 mA and 12 °C. The sample flow rate was 2 mL/h, and the separation buffer flow rate was 4 mL/fraction·h with 90 fractions being collected simultaneously. One 100-mL detergent extract (about 140 mg of protein) was used per FFE run. Prior to FFE, the extracts were concentrated by ultrafiltration (PM10 membrane, Amicon) to about 10 mL and washed with the respective separation chamber buffer until the conductivity reached 1.0–1.5 mS/cm. Depending on the state of the starting material and the running conditions, three different types of FFE runs were performed: (1, type 1) a run in the absence of EDTA and with ammonium acetate/ammonia as separation chamber buffer (1.0 mS/cm, pH 7.0) of a porin-enriched membrane extract; (2, type 2) a run in the presence of 3 mM EDTA and with ammonium acetate or 5 mM sodium phosphate (approximately 1.3 mS/cm, pH 7.6) as separation electrolyte using prepurified material via a type 1 run; (3, type 3) a run in the presence of 3 mM EDTA in ammonium acetate buffer starting with a porin-enriched extract. All buffers contained 2 mM DTT and 0.5% (for type 2 runs) or 1% (for type 1 or type 3 runs) octyl-POE. FFE runs were monitored by measuring the extinction at 280 nm across the terminal part of the separation chamber by means of a photoelectric scanner.

Total protein concentrations were determined by employing the differential extinction at 260/280 nm (Warburg &

Christian, 1942). Porin concentrations were measured at 276 nm where E_{1cm} = 1.41 corresponds to 1 mg/mL (Rosenbusch, 1974).

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate (SDS–PAGE). Discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed after the procedure of Laemmli (1970) using 11 or 12.5% mini (90 × 60 × 0.6 mm) or standard size (165 × 90 × 1 mm) slab gels with a 4.5% stacking gel. Samples containing 3–12 μg of protein were mixed in a 4:1 ratio with sample buffer (pH 6.8) containing 0.5 M Tris, 5% SDS, 5% glycerol, 5% β-mercaptoethanol, and 0.5% bromophenol blue. For runs under "denaturing" (i.e., dissociation of porin trimers and quantitative removal of LPS) conditions, samples were boiled for 5 min prior to loading. For runs aimed at maintaining the trimeric state of porin with most of the LPS still bound, samples were run unboiled. Gels were either stained with Coomassie brilliant blue R-250 (6–12 μg of protein/mini-gel lane) or silver stained (3–6 μg of protein/mini-gel lane) as described by Wray et al. (1981). For standard size gels, twice the amount of protein was loaded.

Analytical Ultracentrifugation. Sedimentation velocity and sedimentation equilibrium runs were performed on a Beckman Spinco Model E analytical ultracentrifuge equipped with absorption optics and a photoelectric scanning system. Sedimentation velocity runs were carried out in an An-D rotor at 52000 rpm and 20 °C, using a 12-mm double-sector Epon cell. For sedimentation equilibrium runs the same cell type was employed but only filled with 0.11 mL of solution and spun in an An-G rotor at 10000 rpm and 20 °C. Protein concentrations used were 0.20, 0.14, or 0.08 mg of porin/mL (in 0.1 M sodium phosphate and 0.1 M NaCl, pH 7.0) in the presence of 1% octyl-POE. This relatively high ionic strength buffer was used to compensate for the possible effect of charge on the sedimentation behavior of the LPS-containing porin trimers (Alexandrowicz & Daniel, 1963). Scanner tracings were taken at 280 nm during both sedimentation equilibrium and sedimentation velocity runs. Molecular masses (M_r) and sedimentation coefficients ($s_{20,w}$) were determined according to the method of Schachman (1959). A value of 0.722 mL/g was used as the partial specific volume of porin as calculated from its amino acid composition (Rosenbusch, 1974); the buffer density was taken as 1.0 g/mL and the buffer viscosity as 1.1 cP. For the calculation of molecular masses, linear regressison (i.e., of the ln A vs r^2 plots; A, absorption at 280 nm; r, distance of the protein in the cell from the rotor axis) and base-line determination programs (written by H. Berger and A. Lustig, Biocenter, Basel) were employed.

Reconstitution of Porin Membranes and Electron Microscopy. Porin was reconstituted into regular membranes according to the method of Dorset et al. (1983) using L-α-dimyristoylphosphatidylcholine (DMPC) as the lipid component. Specimens for electron microscopy were prepared from unfixed samples of reconstituted porin membranes as described (Dorset et al., 1983); however, an aqueous solution of 0.75% uranyl formate, pH 4.25, was used for negative staining. The samples were examined in a Hitachi H-7000 transmission electron microscope equipped with a low-dose kit at an acceleration voltage of 100 kV. Micrographs were recorded under low-dose conditions (~2000 e^-/nm^2) at 50000× nominal magnification and recorded on Kodak SO-163 electron image sheet film. Magnification was calibrated by using negatively stained catalase crystals (Wrigley, 1968). Micrographs containing ordered 2D membrane arrays were analyzed by optical diffraction.

Crystallization. 3D crystals were grown under conditions described elsewhere (Garavito & Rosenbusch, 1986) employing a microdiffusion-in-capillaries technique that was carried out in 25-μL micropipets (Eiselé, 1988).

Results

Preparation of crude *E. coli* membranes involved addition of EDTA to unbroken cells to destabilize the outer membrane (Nikaido & Vaara, 1985) and facilitate the extraction without stripping off any LPS specifically bound to porin [cf. Garavito and Rosenbusch (1986)]. Regarding the addition of EDTA at a later stage, two strategies were followed. If pure porin with a maximum LPS content was desired, any further addition of EDTA was completely omitted. If, on the other hand, a dissociation of LPS was desirable, EDTA was added at a stage where porin was already detergent solubilized. The solubilization of porin involved fractional extraction of the crude membrane preparations with buffers containing 0.5% or 1% octyl-POE, thereby yielding porin-enriched detergent extracts. Applying the modifications described under Materials and Methods to the original procedure (Garavito & Rosenbusch, 1986) confined approximately 70% of the porin yield to two 100-mL extracts. Three factors were found to critically affect the extraction behavior of porin: (1) the extraction buffer to membrane pellet ratio (i.e., milliliters of extraction buffer per gram of crude membrane pellet); (2) the high-speed centrifugation steps; and (3) thorough but gentle homogenization using a Teflon/glass homogenizer. We found that the optimal buffer-to-pellet ratio was 100 mL of extraction buffer for resuspending a pellet of 85–90 g of cells (wet weight). A higher ratio led to concomitant solubilization of additional membrane proteins, whereas, with a lower ratio, solubilization of porin was retarded. Under optimal conditions, a large fraction of the impurities consisting of different membrane proteins was removed during extraction steps 1–8 in a buffer containing 0.5% octyl-POE. Quantitative extraction of porin trimer–detergent micelles was obtained during extraction steps 9–12 for which the octyl-POE concentration was raised to 1%.

Continuous FFE, separating proteins mainly according to their electrophoretic mobilities, was attempted to the earliest possible stage, thereby omitting any of those EDTA/ethanol precipitation steps employed in the procedure detailed by Garavito and Rosenbusch (1986). Following these constraints, the extracts were subjected to FFE after a combined concentration–washing step (see Materials and Methods). For the sake of reproducibility all extraction volumes were standardized since concentration of porin-containing extracts by ultrafiltration led to an increase of the octyl-POE concentration. Usually, the 100-mL extracts obtained during the fractional extraction steps 9 or 10 (see Materials and Methods) were best suited for preparative FFE runs since they contained the highest amount of porin. Extracts 11, 12, etc. also contained porin, but enriched to a lesser extent and present in a much lower concentration. With the exception of the yield, there is, however, no difference between porin purified from extracts 9 versus that obtained from extracts 12. In previous procedures all porin-enriched extracts were pooled and subjected to further purification (Garavito & Rosenbusch, 1986). After a 4-h FFE run (a typical separation profile is displayed in Figure 1) during 3 h of which fractions were collected, the major peak was typically sampled in 10 12-mL fractions. Within the first four to five peak fractions at least 25 mg of more than 95% pure porin was obtained (Figure 2b). Since the porin moiety within the cell extracts used for FFE accounted for approximately 40% of the total protein (Figure

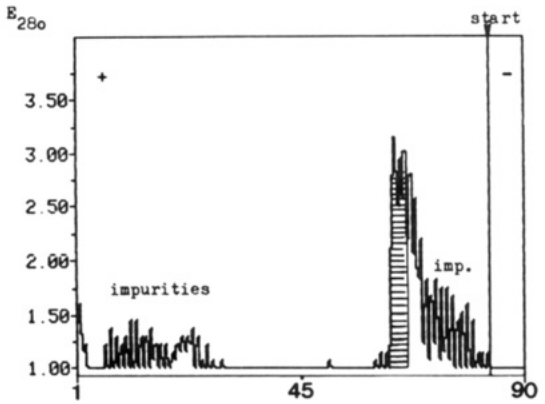

FIGURE 1: Type 1 FFE run: Representative protein separation profile across the terminal part of the separation chamber of a porin-enriched *E. coli* membrane extract at steady state. The *x* coordinates correspond to the fraction numbers. While the more slowly migrating peak fractions (i.e., those on the right side of the peak) contain impure porin, both the fastest and the slowest migrating fractions are devoid of porin and contain the various contaminants of the extract. While the exact position of the major peak depended on the type of run performed, the purest porin was usually found in the fastest migrating fractions on the major peak (hatched area). For type 2 and type 3 runs (see text) performed in the presence of EDTA, the porin free of loosely bound LPS migrated the fastest, while the species with increasing amounts of bound LPS exhibited an increasingly lower mobility (see Figure 3).

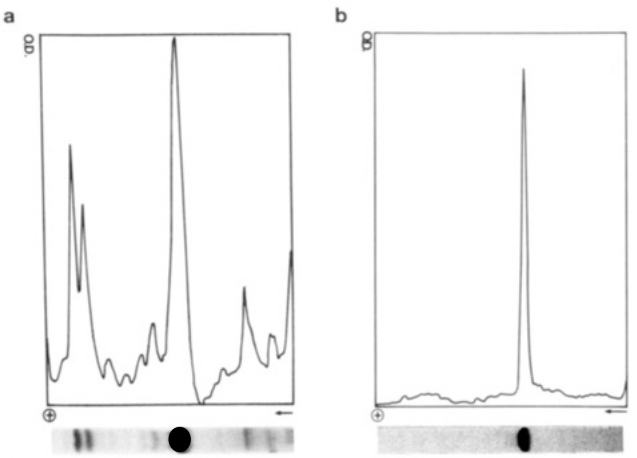

FIGURE 2: Coomassie blue stained SDS–polyacrylamide gel lanes of boiled (i.e., heat denatured) samples together with corresponding densitometer tracings of (a) a porin-enriched *E. coli* membrane extract (with 1% octyl-POE) and (b) purified porin after a single-step FFE run.

2a), i.e., 56 mg of porin/140 mg of protein, its final total yield (i.e., fractions 62–65 in Figure 3) typically amounted to about 45%. Reelectrophoresing under the same conditions increased the final yield toward 80%. As stated above, the resulting porin fractions contained less than 5% impurities as determined by scanning of Coomassie blue stained gels (Figure 2b) and supported by silver stained gels (Figure 4). Since pure porin could be obtained in a single step after fractional extraction of the crude membrane fractions, an increase of the primary yield at the expense of reproducibility and time did not seem to be worthwhile.

We have found that the best compromise between effective protein separation and an acceptable yield of pure porin by FFE was achieved by keeping the protein concentration of the starting material below 20 mg/mL and the octyl-POE concentration in the separation buffer between 0.5% and 1%. With higher octyl-POE concentrations (2%) protein separation was reduced, resulting in a 30% lower yield of pure porin.

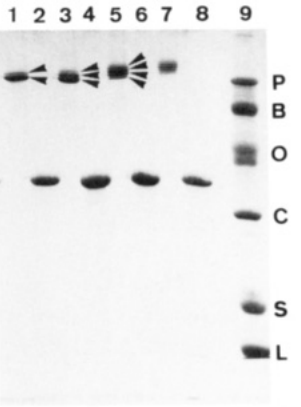

FIGURE 3: 12.5% SDS–polyacrylamide gel stained with Coomassie brilliant blue R-250. Four major peak fractions obtained from a type 3 FFE run displaying porin species containing different amounts of LPS (^{lb}LPS stands for the amount of loosely bound LPS per porin trimer; an asterisk indicates 1 mol and the plus corresponds to 8 mol; see also text) are depicted. In lane 1 two bands (porin ^{*lb}LPS, fraction 62), in lane 3 three bands (porin ^{**lb}LPS, fraction 63), and in lanes 5 and 7 at least four bands (corresponding to porin $^{+lb}$LPS when obtained in the absence of EDTA, fractions 64 and 65) are observed (see arrowheads). The same patterns were revealed after silver staining (not shown). Samples in lanes 1, 3, 5, and 7 are unboiled, whereas lanes 2, 4, 6, and 8 are the same samples but heat denatured. Molecular weight standards are given in lane 9: P = phosphorylase B, 92 500; B = bovine serum albumin, 66 000; O = ovalbumin, 45 000; C = carbonic anhydrase, 31 000; S = soybean trypsin inhibitor, 21 500; and L = lysozyme, 14 000.

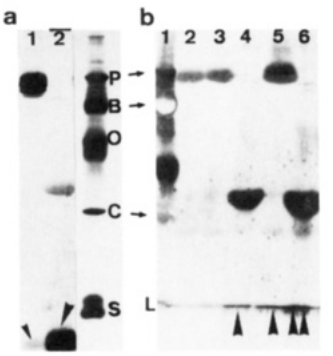

FIGURE 4: Silver-stained 12.5% standard (a) and 11% mini (b) SDS–polyacrylamide slab gels loaded with pure porin samples obtained from type 1 (a) or type 2 (b) FFE runs. (a) Type 1 single-step FFE purified porin $^{+lb}$LPS, (lane 1) unboiled and (lane 2) boiled. The LPS moieties visible at the dye front in the case of the unboiled sample (small arrowhead) are more pronounced after boiling the sample (large arrowhead). Gel standards are as in Figure 3. (b) (Lane 1) Molecular weight standards; (lane 2) porin $^{-lb}$LPS (fraction 65, unboiled); (lane 3) porin $^{-lb}$LPS (fraction 66, unboiled); (lane 4) porin $^{-lb}$LPS (fraction 66, boiled); (lane 5) porin ^{**lb}LPS (fraction 67, unboiled); (lane 6) porin ^{**lb}LPS (fraction 67, boiled). To detect even small amounts of LPS at the dye front, electrophoresis was stopped before the dye front had reached the end of the gel, and the silver staining was overdeveloped. LPS bands appearing at the dye front are marked with arrowheads. Porin $^{-lb}$LPS usually exhibits a LPS band at the dye front only after heat denaturation (lane 4), whereas porin ^{**lb}LPS already reveals a clear band without boiling the sample (lane 5).

The FFE specifications detailed under Materials and Methods fulfilled divergent requirements: type 1 runs (i.e., in the absence of EDTA) were mainly aimed at retaining the full LPS complement (Figure 4a); type 2 runs (i.e., in the presence of EDTA starting with prepurified material) were aimed at a complete removal of loosely bound LPS (Figure 4b); and type 3 runs (i.e., in the presence of EDTA with porin-enriched extracts as the starting material) were aimed at yielding fractions containing porin trimers with different amounts of loosely bound LPS (Figure 3). For type 2 and type

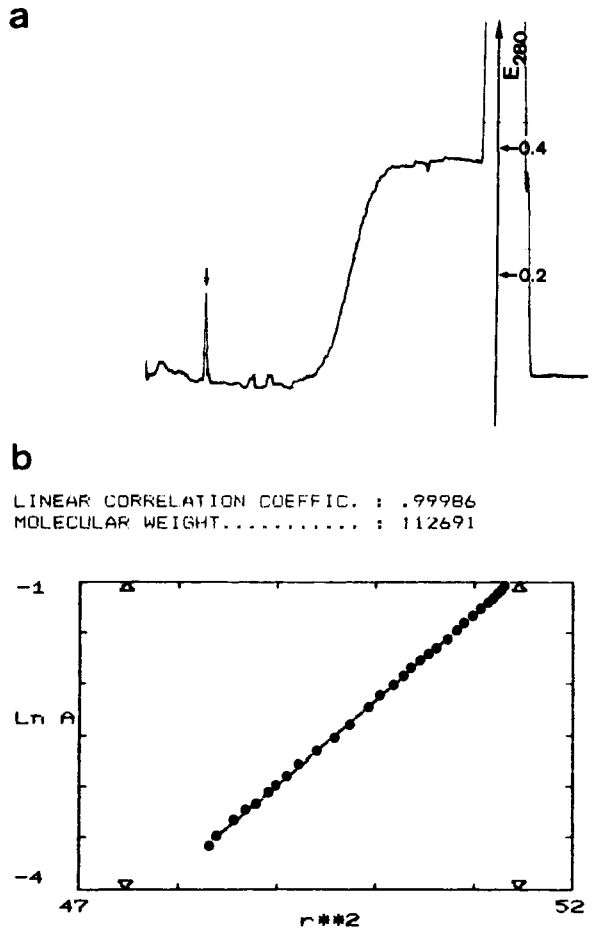

FIGURE 5: Analytical ultracentrifugation of pure porin $^{-lb}$LPS. (a) Representative scanner tracing of a sedimentation velocity run after 80 min at 52 000 rpm. The position of the meniscus peak is marked by an arrow. The shape of the scan is typical for a homogeneous sample without aggregates. (b) ln A vs r^2 plot of a 10 000 rpm sedimentation equilibrium run of porin $^{-}$LPS in the presence of 1% octyl-POE (A, absorption at 280 nm; r, distance of the protein in the cell from the rotor axis).

3 runs, 3 mM EDTA was sufficient to at least partially remove LPS, yet low enough to keep the conductivity of the buffer below 1.5 mS/cm, a prerequisite for high-performance FFE runs. With both type 2 and type 3 runs similar porin–LPS patterns were observed (for a typical pattern see Figure 3). While completely LPS-free porin was never obtained under type 2 conditions, at least 25% of the pure porin fractions were free of loosely bound LPS as judged from silver-stained polyacrylamide gels of unboiled samples, since these revealed a single band only (Figure 4b; see also Discussion). These fractions were therefore referred to as porin $^{-lb}$LPS. Apart from the presence of EDTA, the LPS content of FFE-purified porin was found to depend on the purity of porin in the starting material: the purer the starting material, the higher the yield of porin $^{-lb}$LPS.

Three further porin–LPS isoforms that could also be clearly distinguished by SDS–PAGE of unboiled samples were isolated by employing type 1 and type 3 FFE runs. Fractions revealing 2 (Figure 3, lane 1), 3 (Figure 3, lane 3), or ≥4 bands (Figure 3, lane 5; Figure 4a, lane 1) were observed. Experiments in which isolated LPS molecules were bound to essentially LPS-free porin in known stoichiometric ratios revealed that two bands appeared on Coomassie blue stained polyacrylamide gel if 1 mol of LPS was loosely bound per trimer and that a "ladderlike" bandpattern was obtained after saturating amounts of LPS (10 mol of LPS/mol of porin trimers) were added (L. Bühler, Biocenter, unpublished results). Taking this into account, we herein refer to the "two-band porin" as porin ^{*lb}LPS, and the "three-band porin" as porin ^{**lb}LPS implying that 1 and 2 mol ^{lb}LPS, respectively, were bound per porin trimer. Porin exhibiting ≥4 bands (≥3 mol of ^{lb}LPS/trimer) was referred to as porin $^{+lb}$LPS when obtained via a type 1 FFE run (Figure 4a, lane 1), i.e., in the absence of EDTA. In this case, it was not always possible to unambiguously evaluate the exact number of bands.

Table I: Summary of Analytical Ultracentrifugation Data (Averages) and Linear Regression Analysis

porin–LPS isoform	obsd M_r	calcd LPS content in mol of LPS/porin trimer: total LPS	calcd LPS content in mol of LPS/porin trimer: ^{lb}LPS	predicted M_r using 4500/LPS
porin	nd	0	0	111.6[a]
porin $^{-lb}$LPS	114	0.7	0	114.7
porin ^{*lb}LPS	120	1.9	1.2	119.2
porin ^{**lb}LPS	124	2.7	2.0	123.7
porin $^{+lb}$LPS	151	8.7	8.0	150.7

[a] Polypeptide exclusively according to Chen et al. (1982).

Analytical ultracentrifugation experiments (Figure 5) revealed an overall molecular mass of 114 000 ± 3000 and a sedimentation coefficient $s_{20,w}$ of 5.0 S for porin $^{-lb}$LPS. The sedimentation behavior (Figure 5a) indicated a high degree of homogeneity of the material, i.e., the absence of significant amounts of porin oligomers or aggregates. Analytical ultracentrifugation therefore allowed us to determine whether the multiple bands seen on SDS–PAGE were a product of varying degrees of SDS–PAGE-induced dissociation of LPS from porin or whether the material loaded onto the gels was itself inhomogeneous with regard to the LPS–porin stoichiometry. The ln A/r^2 plots for each of the four species exhibited excellent linearity (correlation coefficients > 0.99), revealing the monodisperse character of the different porin–LPS isoforms isolated by FFE. A summary of the molecular masses found for the different porin isoforms as well as the number of LPS molecules bound is given in Table I. In conjunction with SDS–PAGE and assuming that the 151-kDa isoform has eight mol of ^{lb}LPS bound per trimer (since with this number the best fit was obtained), the results from analytical ultracentrifugation linear regression of mass versus number of LPS molecules (# LPS) gave $M_r = 4.5 \times$ # LPS + 114.7 with an r^2 value of 0.999. This suggests the molecular mass of one molecule of ^{lb}LPS to be 4500. Although analytical ultracentrifugation revealed the different molecular masses of the isolated porin–LPS isoforms, the sedimentation coefficients did not significantly change. Between porin $^{-lb}$LPS, porin ^{*lb}LPS, porin ^{**lb}LPS, and porin $^{+lb}$LPS the $s_{20,w}$ increased from 5.0 to 5.2 S only.

Reconstitution experiments carried out with porin $^{+lb}$LPS and DMPC revealed a characteristic polymorphism where three different protein–lipid lattices could be distinguished: (1) a *large hexagonal* ($p3$) lattice with $a = 9.2 \pm 0.2$ nm and $\alpha = 60°$; (2) a *small hexagonal* ($p3$) lattice with $a = 8.0 \pm 0.2$ nm and $\alpha = 60°$; and (3) a *rectangular* ($p2$) lattice with $a = 8.0 \pm 0.2$ nm, $b = 13.8 \pm 0.2$ nm, and $\alpha = 90°$. On average, ordered membrane patches were 1–2 μm in diameter.

3D porin crystals suitable for X-ray diffraction analysis are a prerequisite to determine the porin structure at near-atomic resolution. Therefore it was of interest whether FFE-purified porin would readily form 3D crystals under conditions that have already been optimized for conventionally purified porin (Garavito & Rosenbusch, 1986). In keeping with previous experience that LPS negatively affects the 3D crystallization (Rosenbusch et al., 1982), we used FFE-purified porin $^{-lb}$LPS

for crystallization experiments. Within 4–6 days crystals could be grown that exhibited a polymorphism very similar to that reported by Garavito and Rosenbusch (1986).

Discussion

FFE has already proven its potential for gentle separation of biological material for almost 40 years (Svensson & Brattsten, 1949; Grassmann, 1951). For instance, it has been successfully used to isolate immunocompetent lymphocytes without vitality loss, to separate "inside-out" vesicles from "outside-out" vesicles (Hannig, 1978), and to purify a number of membranous compounds (Hannig & Heidrich, 1974). Purification of the tissue plasminogen activator, a glycosylated membrane protein, by FFE has yielded a 30–50% higher recovery of activity than obtained with immunoaffinity methods (Barth et al., 1986), and the purification of clathrin-coated vesicles by FFE provided purer material than by permeation chromatography. The latter results have been very recently reported by Morris et al. (1988). Also, it has been shown that FFE is an effective tool for rapid separation of extracted LPS from nucleic acids (Kuwae & Kurata, 1984).

For both structural and functional studies, it is crucial to devise a purification scheme that yields active porin trimers. As uncertainties still remain regarding the minimal amount of LPS required for the biological activity of porin, a gradual removal of the bound LPS should be attempted, a prerequisite for studies that ultimately will reveal the functional role of LPS in channel activity. In this view, our approach complements previously published purification methods aimed at isolating LPS-free porin (Garavito & Rosenbusch, 1986; Worobec et al., 1988). The purification procedure by means of FFE presented here allows separation into different porin–LPS isoforms. The total yields of pure LPS-containing porin in a single type 1 or type 3 run were at least 45% and may be further improved by repeated passes of partially purified porin fractions since the overall recovery is close to 100% in each run.

The four porin–LPS isoforms separated by FFE were characterized (1) by SDS–PAGE and (2) by analytical ultracentrifugation. SDS–PAGE of boiled and unboiled porin–LPS complexes indicates that there are loosely as well as tightly bound LPS molecules, the latter dissociating under denaturing conditions (e.g., boiling) only [cf. Rocque et al. (1987)]. This was most clearly visible from SDS–PAGE of the porin–LPS isoform free of loosely bound LPS, which gives rise to a single sharp band without boiling (Figure 4b, lane 2 or 3). After boiling, the same sample exhibited a distinct leading band due to the LPS moiety migrating near the dye front (Rocque et al., 1987) in addition to the band of porin monomers at about 37 kDa (Figure 4b, lane 4). Similarly, the intensity of the leading band markedly increases (Figure 4b, lane 6) after boiling of porin containing loosely bound LPS. Loosely bound LPS, on the other hand, partially dissociated during SDS–PAGE even without boiling, yielding two or more closely spaced bands ("ladders"; see Figure 3) together with a leading band corresponding to the LPS moiety (Figure 4, lane 5). In this context it should be noted that LPS moieties can be clearly visualized by the silver-staining method of Wray et al. (1981) without employing the more LPS-sensitive silver stain described by Tsai and Frasch (1982). Unboiled porin–LPS isoforms giving rise to a ladder of bands of SDS–PAGE appeared monodisperse as revealed by analytical ultracentrifugation, indicating that in solution there is just one isoform present. This suggests that FFE allows porin–LPS complexes to be separated according to their number of LPS molecules bound.

The molecular masses of different porin–LPS isoforms determined by analytical ultracentrifugation fitted by linear regression as a function of the putative number of loosely bound LPS molecules yielded an LPS molecular mass of 4500, which is in excellent agreement with published data (4300; Mizushima, 1985). This finding supports our interpretation of the four porin–LPS species isolated by FFE (Table I) and justifies the proposed nomenclature: i.e., $^{-lb}$LPS (no loosely bound LPS); ^{*lb}LPS (1 mol of loosely bound LPS per trimer); ^{**lb}LPS (2 mol of loosely bound LPS per trimer); $^{+lb}$LPS (8 mol of loosely bound LPS per trimer). Furthermore, our ultracentrifugation data suggest that the maximum number of LPS molecules bound per porin trimer, including approximately 1 mol of tightly bound LPS, is nine. The same value has been previously reported by Rosenbusch et al. (1980). However, since all these investigations were carried out with *E. coli* B^E strains, it remains still a matter of speculation whether the same value is also applicable to *E. coli* K-12. For the OmpF proteins from K-12 and B strains it is, for instance, known that they only vary in three amino acids [positions 66, 117, and 262; B: Glu, Gly, Leu (Chen et al., 1982); K-12: Gln, Glu, Gln (Inokuchi et al., 1982)] while, on the other hand, the chemical structures of K-12– and B–LPS are considerably different (Prehm et al., 1975, 1976). This is particularly true regarding the number of negatively charged groups, which are most probably the sites of protein–LPS interaction [cf. Lugtenberg and Van Alphen (1983)].

The reconstitution experiments presented here were all carried out by using porin $^{+lb}$LPS. The three different 2D lattices that have been distinguished represent a characteristic feature already observed with conventionally purified porin, presumably porin $^{-lb}$LPS (Dorset et al., 1983). The projection symmetries and center-to-center distances within the unit cells closely match those determined previously (Dorset et al., 1983), suggesting a location of the LPS molecules within the porin trimer that does not interfere with the arrangement of the porin–porin contacts specifying the 2D lattice. Thus, the LPS molecules could extend approximately normal to the membrane plane.

Ultimately the FFE-purified porin isoforms containing different amounts of LPS may enable to evaluate the minimal amount of LPS necessary for porin to retain its channel activity (Schindler & Rosenbusch, 1981). Recent experiments employing a newly designed dialysis device (Holzenburg et al., 1988) revealed highly ordered porin–lipid sheets large enough (i.e., >5 μm in diameter) for functional studies, i.e., patch-clamping, to be performed. At the same time the $^{-lb}$LPS fractions are suitable for the growth of X-ray-quality 3D crystals. Structural investigations concomitant with functional studies employing different porin–LPS species will be useful to learn more about the porin–LPS interactions and to understand at the molecular level the actual role of each part in the regulation of the porin channel permeability. Furthermore, the different isolated porin–LPS isoforms may be an important tool to prove the hypothesis that the binding of LPS to porin, possibly corresponding to the eukaryotic glycosylation process, assists in the proper insertion of porin into the outer membrane (Pages & Bolla, 1988).

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