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IDENTIFICATION OF THE PROTEIN PRODUCING TRANSMEMBRANE DIFFUSION PORES IN THE OUTER MEMBRANE OF *PSEUDOMONAS AERUGINOSA* PA01 *

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Summary

The outer membrane of *Pseudomonas aeruginosa* PA01 is permeable to saccharides of molecular weights lower than about 6000. Triton X-100/EDTA-soluble outer membrane proteins were fractionated by ion-exchange chromatography in the presence of Triton X-100 and EDTA, and the protein contents of the various fractions analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Each of the major protein bands present in the Triton X-100/EDTA soluble outer membrane was separated from one another. Adjacent fractions were pooled, concentrated and extensively dialyzed to reduce the Triton X-100 concentration. Vesicles were reconstituted from lipopolysaccharide, phospholipids and each of these dialyzed fractions, and examined for their ability to retain [^{14}C]sucrose. Control experiments indicated that the residual levels of Triton X-100 remaining in the dialyzed fractions had no effect on the formation or permeability to saccharides of the reconstituted vesicles. It was concluded that a major outer membrane polypeptide with an apparent weight of 35 000 is a porin, responsible for the size-dependent permeability of the outer membrane.

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* This is paper XX in the series on the outer membrane of Gram negative bacteria. The preceding paper is Ref. 8.

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Introduction

The outer membrane of a number of gram-negative bacteria is a size-dependent permeability barrier. Thus only the hydrophilic molecules below a finite molecular size are allowed to diffuse passively through the membrane. The outer membranes of the enteric bacteria *Escherichia coli* K12 and B, *Salmonella typhimurium*, *Proteus mirabilis*, and *Proteus morganii* have an exclusion limit for saccharides of about 600 daltons [1—3]. This exclusion limit is reflected by the permeability properties of vesicles reconstituted from the individual components of outer membranes [3,4] including the lipopolysaccharide, phospholipids, and the unique set of outer membrane proteins, and has been shown to be due to one class of proteins named porins [5—7]. The number and nature of the porins varies from strain to strain, but generally they are major outer membrane polypeptides of around 35 kD (kilodaltons).

Studies with *Pseudomonas aeruginosa* revealed that significant levels of larger saccharides, which were excluded by the outer membranes of enteric bacteria, diffused into plasmolysed *P. aeruginosa* cells [1]. Although these data were earlier interpreted as the result of outer membrane damage caused by the stretching of the membrane during plasmolysis [1], fusion of fragments of the outer membrane with phospholipid-lipopolysaccharide vesicles showed that the reconstituted vesicles, which did not suffer from the problem of membrane stretching, still showed a large exclusion limit of 6000 ± 3000 daltons [8]. In view of the observation [4] that the exlusion limit of similar reconstituted vesicles containing outer membrane proteins from *S. typhimurium* accurately matched the exclusion limit of plasmolysed cells of the same strain (which does not become damaged by plasmolysis as severely as does *P. aeruginosa* (Decad, G.M. and Nikaido, H., unpublished results)), we now believe that the true exclusion limit of *P. aeruginosa* outer membrane lies between 3000 and 9000 daltons, and that our previous interpretation [1] of plasmolysed cell data was incorrect.

We have recently introduced a method of separating outer and inner membranes of *P. aeruginosa*. The outer membrane was shown to contain four major protein bands of 17, 21, 35 and 37 kD on SDS polyacrylamide gel electrophoresis. The 35 and 37 kD bands are separated only under certain conditions [8]. In most outer membrane preparations the level of 37 kD protein is low (Hancock, R., unpublished results); the possible relationship between 35 and 37 kD proteins will be mentioned in the Discussion. In this paper we describe a method for separating the 17, 21, and 35 kD proteins one from another, and show that the 35 kD protein is a porin of *P. aeruginosa*.

Materials and Methods

Bacterial strains and outer membrane preparation. P. aeruginosa PA01 was used throughout. Cells were grown and the outer membrane fractions OM1 and OM2 isolated as previously described [8], except that the second sucrose density gradient centrifugation was done in a Beckman SW27 rotor at 96 000 × *g* for 18 h due to the large volumes involved.

Reconstitution of outer membrane vesicles. Vesicles were reconstituted from

purified *P. aeruginosa* PA01 lipopolysaccharide, lipids and outer membrane protein by a slight modification of the techniques of T. Nakae as previously reported [8]. As a source of outer membrane protein, various column fractions (see below) were used instead of OM1 or OM2 band proteins. In order to measure the effect of Triton X-100 on the formation and permeability of reconstituted vesicles, it was added at the same time as lipopolysaccharide during the vesicle reconstitution procedure. In some experiments purified *S. typhimurium* TA1014 porins [7], a gift of Dr. T. Nakae, were reconstituted into vesicles containing *P. aeruginosa* lipopolysaccharide and phospholipids.

Chemicals. The isolation of the various radioactive saccharides used was previously described [8]. In general, most reconstitution experiments were performed using [^{3}H]dextran of 50 kD and [^{14}C]sucrose. Triton X-100 was purchased from Sigma (St. Louis, MO) and DEAE-Sephacel, a beaded ion exchanger, from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

Assays. Triton X-100 concentrations were assayed by the method of Garewal [11] with the modification that only 0.75 ml of ethylene dichloride was used for extraction (instead of 1.5 ml), thus doubling the sensitivity of the technique. Protein was assayed in the presence of Triton X-100 using procedure of Sandermann and Strominger [9]. All other assays were previously described [8].

Fractionation of P. aeruginosa outer membrane proteins. The OM1 and OM2 bands from 15 l of cells grown to an adsorbance at 650 nm of 1.0 were pooled to yield 190 mg of combined outer membrane protein. This was then solubilized at a protein concentration of 10 mg/ml in 2% (v/v) Triton X-100 in 20 mM Tris-HCl buffer, pH 7.4, with 2 min sonication in a bath type sonicator (Bransonic 12, Branson Co., Shelton, CT) to assist solubilization. Insoluble protein was sedimented by centrifugation at 177 000 × *g* for 1 h, and the supernatant (40 mg protein) set aside as Triton-soluble protein. The Triton-insoluble protein was then resuspended in 2% Triton X-100/10 mM EDTA in Tris-HCl buffer at a protein concentration of 8 mg/ml, with sonication as above to assist solubilization. Triton/EDTA insoluble protein (25 mg) was removed by centrifugation at 177 000 × *g* for 1 h. The supernatant, containing Triton/EDTA-soluble outer membrane proteins, was applied to a column (2.7 × 38.5 cm) of DEAE-Sephacel which had been equilibrated with 0.1% (w/v) Triton X-100/10 mM EDTA/Tris-HCl buffer (column buffer), and eluted with column buffer containing various concentrations of NaCl as indicated in Fig. 1. Fractions of 6.7 ml were collected, assayed for protein and adjacent fractions pooled as described below. The pooled column fractions were concentrated to about 3—5 ml by dialysis against a 30% (w/v) solution of polyethyleneglycol 4000 (J.T. Baker, Phillipsburg, NJ), followed by dialysis against 5% (v/v) ethanol in distilled water for 22 days at 4°C. SDS polyacrylamide gel electrophoresis of the samples was performed by the method of Lugtenberg et al. [10].

Results

SDS polyacrylamide gel electrophoresis of the fractionated outer membrane proteins

The pooled fractions from the DEAE Sephacel column (Fig. 1) were

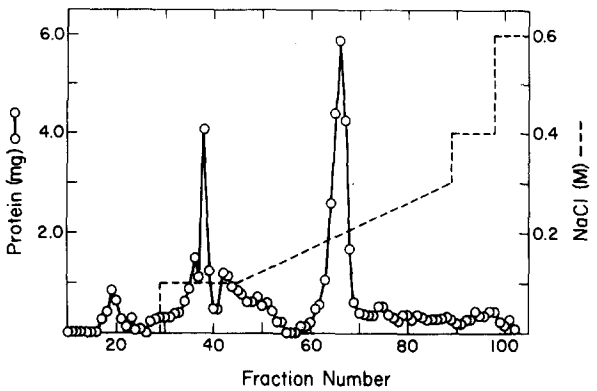

Fig. 1. Separation by DEAE-Sephacel chromatography of Triton X-100/EDTA-soluble outer membrane proteins of *P. aeruginosa* PA01 (○———○). The sample preparation and column buffer are described in Materials and Methods. The column was eluted with varying concentrations of NaCl in column buffer (------). Fractions were pooled, concentrated, dialysed and analysed for protein composition as described in the text and in Fig. 2.

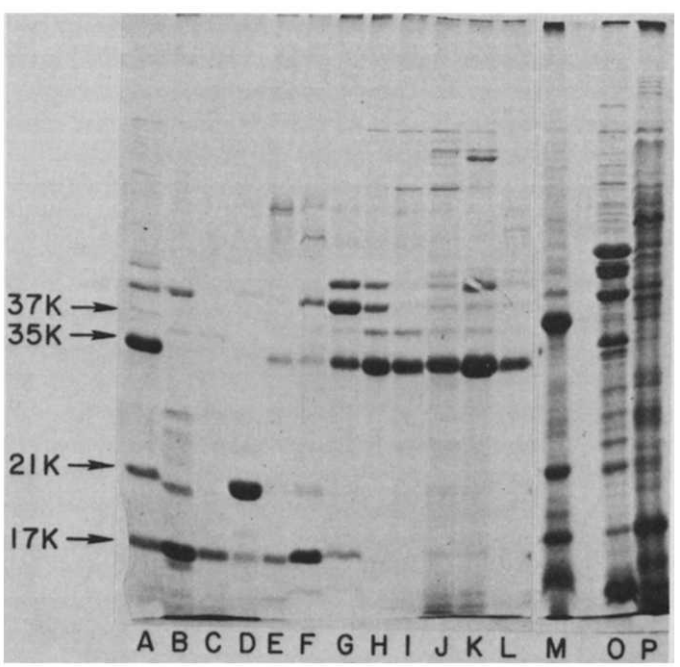

Fig. 2. SDS polyacrylamide gel electrophoresis of *P. aeruginosa* outer membrane proteins fractionated on a DEAE-Sephacel column (Fig. 1). Gel A,Triton/EDTA-soluble outer membrane proteins (this preparation was loaded onto the column in Fig. 1 and fractionated to yield the fractions described below); gel B, fractions 18—21 from the DEAE-Sephacel column; gel C, fractions 34—36; gel D, fractions 38—39; gel E, fractions 42—44; gel F, fractions 46—52; gel G, fractions 61—63; gel H, fractions 64—65; gel I, fractions 66—67; gel J, fractions 68—71; gel K, fractions 73—77; gel L, fractions 90—99; gel M, crude outer membrane extracted with phenol (this causes the 35 kD and 37 kD bands to run as a single band at the position of the 37 kD band: see Ref. 8); gel O, Triton/EDTA-insoluble outer membranes; gel P, Triton-soluble outer membrane. The running positions of the 17, 21, 35 and 37 kD major outer membrane proteins of *P. aeruginosa* PA01 [8] are shown on the left. In the above and other preparations the 37 kD band was not prominent. Approximately equal amounts of protein were loaded onto each gel and thus the gels do not represent the actual amounts of the individual proteins in the fractions (show in Fig. 1) but rather the relative proportions of the various proteins.

analysed by SDS polyacrylamide gel electrophoresis, and compared to the Triton/EDTA-soluble protein which was loaded onto the column and the other fractions derived during the preparation of the protein for chromatography (Fig. 2). The Triton-soluble protein (Fig. 2, gel P) which was discarded contains mainly contaminating inner membrane proteins and significant amounts of only one of the major outer membrane bands, the 17 kD band, which is also prominent in the Triton/EDTA-soluble material (Fig. 2, gel A). The material loaded onto the column, the Triton/EDTA-soluble proteins, contained substantial amounts of the 17, 21 and 35 kD major outer membrane proteins (Fig. 2, gel A). The 37 kD protein observed previously as a major protein [8], was not present in large amounts in this or another similar preparation. Specific fractions were substantially enriched for three of the major outer membrane proteins. Fractions 34—36 (Fig. 2, gel C), 38—39 (Fig. 2, gel D) and 66—67 (Fig. 2 gel I) were rich in the 17, 21 and 35 kD proteins, respectively. Although other column fractions contained the major outer membrane proteins, the above-mentioned fractions were the purest preparations of these proteins obtained in this study and were essentially uncontaminated by the other major bands. Fractions 38—39 and 66—67 were the main peaks from the column (Fig. 1). The 37 kD protein was present as a minor band in all fractions containing the 35 kD protein. Other proteins were prominent in one or more fractions.

Reconstitution of vesicles using fractionated outer membrane proteins

When proteins in the column fractions were precipitated out of Triton X-100 solution with 2 vols. of ethanol, none of the fractions could reconstitute sucrose-permeable vesicles. Therefore, we decided to use dialysis to remove excess Triton X-100. Dialysis for 22 days at 4°C against 5% aqueous ethanol resulted in the removal of 90—95% of the Triton X-100. In order to demonstrate that Triton X-100 did not affect reconstitution, the effect of various concentrations of Triton X-100 on the retention of [^{3}H]dextran and the ratio of [^{3}H]dextran : [^{14}C]sucrose retained, was tested (Table I). These results demonstrated that concentration less than 0.05—0.07% (v/v) Triton X-100 did not reduce the amount of [^{3}H]dextran retained, suggesting that closed vesicles were being formed. The enhancement of incorporation at lower Triton X-100 concentrations was probably caused by the more efficient dispersal of vesicle components prior to vesicle formation. The ratio of [^{3}H]dextran : [^{14}C]sucrose retention was also relatively unaffected by Triton X-100 concentrations lower than 0.05—0.07%, suggesting that at these concentrations the presence of Triton X-100 did not result in enhanced leakage of one saccharide relative to the other. These results were observed both with vesicles made only of lipopolysaccharide and phospholipids, and with vesicles which had incorporated purified *S. typhimurium* porin protein.

From the above results, it was concluded that as long as the Triton X-100 concentration was held below 0.05% (approximately equivalent to 1 mol Triton X-100 per 7 mol of phospholipid), the vesicle reconstitution experiment would reflect only the ability of the added protein fraction to produce sucrose permeable pores, In fact, in most of the experiments reported below, the Triton X-100 concentration was considerably lower.

The results of vesicle reconstitution experiments with the various column

TABLE I

EFFECT OF TRITON X-100 ON THE RECONSTITION OF OUTER MEMBRANE VESICLES AND THE RETENTION BY THE VESICLES OF [^{3}H]DEXTRAN AND [^{14}C]SUCROSE

Vesicles were formed in the presence of labelled saccharides using *P. aeruginosa* PA01 lipopolysaccharide (0.08 μmol) and phospholipids (0.5 μmol) in the presence or absence of purified *S. typhimurium* TA1014 porin protein (130 μg) as previously described [8]. Triton X-100, when included, was added at the same time as lipopolysaccharide during vesicle formation. Percent [^{3}H]dextran incorporated is the percentage of input [^{3}H]dextran (of 50 000 daltons) passively enclosed during vesicle formation. After vesicle formation, the extravesicular medium was diluted 100-fold and incubated for 20 min at 23°C to allow maximum possible leakage of saccharides out of the vesicles. Further incubation did not alter the results shown.

Triton X-100 added (% v/v)	Percent [^{3}H]dextran incorporated		Ratio [^{3}H]dextran: [^{14}C]sucrose *	
	+porin	No porin	+porin	No porin
0	3.0	2.1	52	3.2
0.01	4.5	3.5	45	3.5
0.025	4.5	3.1	54	3.3
0.05	4.0	3.2	40	2.6
0.07	3.4	3.0	35	1.9
0.10	2.2	1.4	20	1.6
0.20	— **	0.6	— **	1.3

* In the presence of *S. typhimurium* porin, preferential leakage of [^{14}C]-sucrose occurred resulting in a higher ratio of [^{3}H]dextran: [^{14}C]sucrose.

** Not done.

fractions are shown in Table II, in which the extent of formation of vesicles permeable to sucrose but not to dextran is indicated by the magnitude of the 'permeability index'. As a standard, Triton/EDTA-soluble outer membrane proteins were reconstituted into vesicles. This resulted in a moderate increase in permeability to sucrose. In contrast, the fractions 34—36 and 38—39, enriched in the 17 and 21 kD major outer membrane proteins, respectively (Fig. 2, gels C and D), caused only a very small increase in sucrose permeability even at quite high concentrations. Most of the other fractions contained porin activity as judged by the enhanced sucrose permeability resulting from small amounts of protein. In each of these fractions the most prominent protein was the 35 kD protein. The only other band consistently present in all fractions with porin activity was the 37 kD band. The major peak from the column, represented by fractions 66—67, contained only four protein bands (Fig. 2, gel I); the 35 kD band comprised over 80% of the protein in these fractions. 5 μg of proteins from this fraction, when reconstituted into vesicles, resulted in a higher permeability index than 20 μg of Triton/EDTA-soluble outer membrane proteins. Although other protein bands were present in all fractions with porin activity, there was no correlation between porin activity and the presence of any proteins other than the 35 and 37 kD proteins.

Vesicles were reconstituted from lipopolysaccharide, phospholipids and 15 μg of fraction 66—67 in the presence of [^{14}C]sucrose and [^{3}H]saccharides of various molecular weights. Only those saccharides of average molecular weight greater than 9000 were fully retained by these vesicles (Fig. 3).

TABLE II

RECONSTRUCTION OF THE OUTER MEMBRANE PERMEABILITY BARRIER IN VESICLES USING FRACTIONATED OUTER MEMBRANE PROTEINS FROM A DEAE-SEPHACEL COLUMN

The ratios of [^{3}H] dextran: [^{14}C] sucrose, retained in vesicles reconstituted from lipopolysaccharide and phospholipids in the presence or absence of the indicated amounts of protein derived from the various pooled column fractions (Figs. 1 and 2), were measured. In order to correct for the amounts of Triton X-100 associated with the column fractions, an equivalent amount of Triton X-100 was added to the control vesicles formed only from lipopolysaccharide and phospholipids. The permeability index was determined using the following formula:

$$\text{Permeability index} = \frac{\text{Ratio of } [^3\text{H}]\text{dextran: } [^{14}\text{C}]\text{sucrose retained in the presence of added protein}}{\text{Ratio of } [^3\text{H}]\text{dextran: } [^{14}\text{C}]\text{sucrose retained in the absence of added protein}}$$

The presence of a porin leads to specific leakage of [^{14}C] sucrose out of the vesicles, resulting in an enhanced [^{3}H] dextran: [^{14}C] sucrose ratio and therefore a higher permeability index. Thus a permeability index of one would indicate no enhancement of sucrose permeability. As a comparison 130 μg of unfractionated *P. aeruginosa* PA01 outer membrane protein gave a permeability index of 5.55 to 6.25 (derived from the results presented in Ref. 8).

Fraction No.	Triton X-100 (μg/μg protein)	Permeability index					
		5 μg	10 μg	15 μg	20 μg	30 μg	50 μg
34—36	0.30	—*	—	—	—	1.1	1.6
38—39	0.84	—	—	—	1.2	1.3	
42—44	0.63	—	—	—	3.7	4.0	—
46—52	4.40	—	1.7	—	—	—	—
61—63	2.03	—	1.4	—	1.8	—	—
64—65	0.62	1.5	2.3	2.6	—	—	—
66—67	1.11	2.7	3.6	4.6	6.0	6.2	—
68—71	2.27	1.9	2.8	—	5.5	—	—
73—77	4.64	—	3.4	—	—	—	—
90—99	0.21	2.6	3.8	—	6.3	—	—
Triton/EDTA-soluble	0.21	—	1.7	—	2.5	—	—

* Not done.

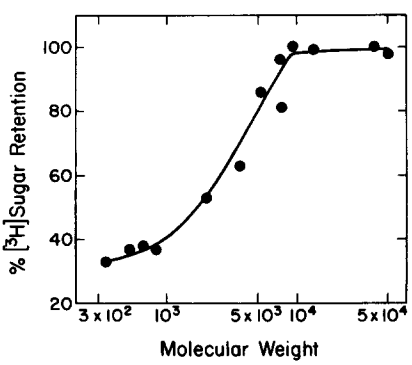

Fig. 3. Exclusion limit for saccharides (both inulins and dextrans, see Ref. 8) of vesicles reconstituted from lipopolysaccharide, phospholipids and 15 μg of fraction 66—67. For each saccharide, control vesicles were formed from *P. aeruginosa* PA01 lipopolysaccharide and phospholipids in the presence of the appropriate amount of Triton X-100 (i.e., that amount present in association with fraction 66—67; 16 μg). It was shown for each saccharide that the Triton X-100 had little effect on the incorporation of saccharides and thus retention by these control vesicles was taken as 100%. The retention of saccharides by vesicles which had incorporated partially purified porin protein (fraction 66—67, see text), was expressed relative to the retention by control vesicles.

Discussion

There is a correlation between the presence of the 35 kD (and 37 kD) protein in the various column fractions and the ability of these fractions to form sucrose-permeable pores. In fact, as shown in Fig. 3, the pores formed in reconstituted vesicles by the 35 kD protein appear to have the same exclusion limit as unfractionated outer membrane [8]. Neither the 17 kD nor the 21 kD major outer membrane proteins formed sucrose permeable pores in reconstitution experiments, nor were correlations between the presence of other more major outer membrane proteins and porin activity observed. Since there is a large excess of 35 kD protein over 37 kD in all of the fractions which can form sucrose permeable pores, we conclude that the 35 kD protein is probably a porin, while the 37 kD protein may also have porin activity. Previous experiments indicated that these proteins are possibly closely related one to another [8], and this is further evidenced by the fact that they cochromatograph upon ion-exchange chromatography. We were unable to separate the two bands by molecular sieving in 1% sodium deoxycholate/10 mM EDTA/Tris-HCl-buffer unpublished results). Unfortunately, SDS destroys irreversibly the ability of outer membrane proteins to reconstitute sucrose-permeable vesicles together with lipopolysaccharide and phospholipids [12], so we could not use this detergent for the purification and separation of porins as it has been used for *S. typhimurium* [5,7] and *E. coli* [6]. One possibility is that the 35 kD protein differs from the 37 kD protein only in that it is bound to lipopolysaccharide, since phenol treatment, which results in the removal of lipopolysaccharide, causes both proteins to run as a single band of 37 kD (Ref. 8; see also Fig. 2, gel M).

The outer membrane hydrophilic pores of *P. aeruginosa* PA01 are larger than those of four enteric bacteria [1—3,8] the exclusion limit being of the order of 6000 ± 3000 daltons compared to a figure of about 600 daltons for enteric bacteria. The partially purified porin of *P. aeruginosa* forms a pore with the same exclusion limit as whole outer membranes. Thus it would seem that, like enteric bacteria, a single type of protein results in a hydrophilic pore, despite the very different sizes of the respective pores.

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