IDENTIFICATION OF THE OUTER MEMBRANE PROTEIN OF E. COLI THAT PRODUCES TRANSMEMBRANE CHANNELS IN RECONSTITUTED VESICLE MEMBRANES*

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SUMMARY. Outer membrane of Escherichia coli allows a rapid diffusion of saccharides of molecular weights less than 550. This permeability property could be restored in vesicle membranes reconstituted from isolated phospholipids, lipopolysaccharide, and an outer membrane protein. The active protein aggregates were isolated from the insoluble material left after solubilization of cell envelope of Escherichia coli B with sodium dodecyl sulfate at 35°. Analysis by acrylamide gel electrophoresis, isoelectric focusing and amino terminal amino acid determination revealed that only a single species of protein, with a molecular weight of 36,500 forms the oligoprotein aggregates which produces diffusion channels.

The cell envelope of Gram-negative bacteria consists of cytoplasmic membrane, peptidoglycan and outer membrane. Outer membrane, the outer-most layer, acts as a permeability barrier for larger oligopeptides (1), neutral sugars with molecular weights more than 700 (2, 3) and hydrophobic dyes and antibiotics (4). But the membrane is freely permeable to hydrophilic small molecules such as glucose, sucrose and amino acids.

We have shown (5-7) that the reconstitution of membranes permeable to small hydrophilic molecules requires the presence of an aggregate of outer membrane proteins, in addition to phospholipids and LPS. The active aggregate isolated from Salmonella typhimurium contained three proteins. Here I describe the isolation, from Escherichia coli B, of an aggregate containing only one kind of protein, which confers similar hydrophilic permeability to the reconstituted membranes.

MATERIALS AND METHODS

Bacterial strain. Escherichia coli B was used.

Isoelectric focusing. The procedure used for isoelectric focusing of outer membrane protein was based on the procedure of O'Farrell (8) modified

* This is Paper XI of the series on outer membrane.

Abbreviations: SDS, Sodium dodecyl sulfate; LPS, Lipopolysaccharide

by Ames and Nikaido (9). The total concentration of carrier materials is 2% that comprise the following proportions of pH ranges: Ampholine 3.5-10, Bio-Lite 5-7, and Ampholine 3-5 in the ratio of 1:2:2.

Amino terminal analysis. This was performed with dansyl chloride (10). Dansylated amino acid was identified by thin layer chromatography on polyamide sheets. The solvent systems used were (1) water-90% formic acid (200:3, v/v) for the first dimension, (2) benzene-acetic acid (9:1, v/v) and (3) ethyl-acetate-methanol-acetic acid (20:1:1, v/v) for the second dimension.

SDS-polyacrylamide gel electrophoresis. Three different methods of SDS-gel electrohporesis (11-13) were employed.

Reconstitution of outer membrane vesicles and assay for permeability. These were described previously (6, 7).

Other procedures. Extraction and quantitation of LPS and phospholipids were described previously (5, 6). Preparation of membrane and membrane protein is given in previous publication (7). Methods for the purification of active protein complex and calibration of the Sepharose 4B column were described (7).

Chemicals. Chemicals used were the best grades commercially available.

RESULTS

Isolation of the active protein fraction. Various subfractions containing outer membrane proteins were assayed for permeability-conferming activity by reconstituting membrane vesicles with phospholipids and LPS in the presence of [^{14}C]sucrose and [^{3}H]dextran (6, 7). If sucrose permeable vesicles were formed, [^{14}C]sucrose diffused out of the vesicles during the washing of vesicles on Millipore filter (7), and the normalized $^{3}H/^{14}C$ ratios of washed vesicles became larger than 1. When the cell envelope fraction of *E. coli* B was treated with SDS at 35°, the insoluble residue containing peptidoglycan fragments with a few attached proteins was far more active in channel formation than the SDS-soluble fraction, when tested after removal of SDS (Table I). The proteins attached to the peptidoglycan could be released without reducing the channel-forming activity, by treating the insoluble residue with lysozyme, which degrades peptidoglycan, or trypsin, which hydrolyzes the Braun lipoprotein (14).

Among the four fractions obtained by gel filtration, in SDS, of the trypsin-treated materials (Fig. 1), only Fractions I and II were strongly active (Table I).

Characterization of the active protein. The protein composition of Fraction I through Fraction III was analyzed by SDS-polyacrylamide gel electrophoresis after heating them at 100° in 2% SDS. Fraction I and II showed a heavily stained protein band at a location corresponding to 35,000

Table I. Activities of various protein preparation for formation of sucrose permeable vesicles. Vesicles were reconstituted from 1 μmole of phospholipids, 0.05 μmoles of LPS, and proteins, all from E. coli B. Permeability of vesicles was assayed by [^{14}C]sucrose exit from the vesicles that had preloaded with [^{14}C]sucrose and [^{3}H]dextran (60,000 daltons) (6, 7).

Protein preparation*	Amounts used (μg)	Normalized $^{3}H/^{14}C$ ratio
None	-	1.2
SDS-extractable fraction	200	7
SDS-insoluble fraction	10	6
	50	27
	200	60
Fraction I	10	12
	50	>100
	200	>100
Fraction II	10	10
	50	22
	200	>100
Fraction III	200	7
Fraction IV	200	6

* E. coli B. was grown to late exponential phase in L-broth, SDS-extractable and SDS-insoluble fraction of cell envelope were prepared as reported earlier (7). Fraction I through IV were prepared as described in legend for Fig. 1. All fractions were tested after removal of SDS by exhaustive dialysis.

daltons and trace amounts of a small peptide corresponding to the Braun lipoprotein. Fraction III consisted largely of Braun lipoprotein.

In order to make certain that the 35,000 dalton band in Fraction I really consists of a single species of protein, the following experiments were performed. (i) SDS-acrylamide electrophoresis was performed according to two other procedures, which resolved the "major" protein band of E. coli K12 into several bands (12, 13). The 35,000 daltons band of Fraction I, however,

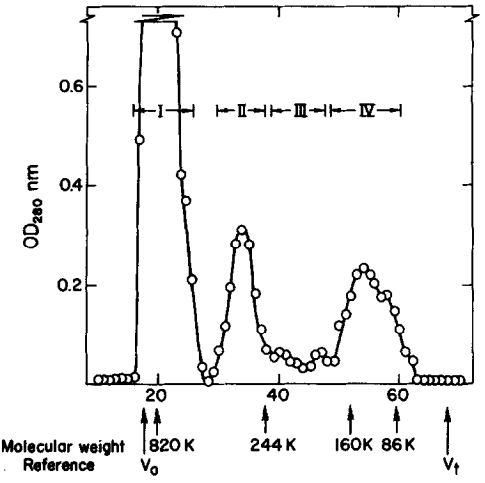

Fig. 1. Gel filtration of protein aggregates obtained from the SDS-insoluble fraction. SDS-insoluble fraction (40 mg protein) of cell envelope, prepared as described in Table I, was suspended in 4 ml of 5 mM Tris-HCl (pH 8.0)-3 mM NaN_3 and was dispersed by sonication (Biosonic IV, 90 sec). The suspension was then incubated for 2 hrs at 37° after the addition of trypsin (400 µg). The sonication and trypsin treatment was repeated two more times, then SDS was added to the final concentration of 2%, and the mixture was kept at 25° for 30 min. The mixture was applied onto a 2.54 x 53 cm Sepharose 4B column equilibrated with 0.1% SDS-5 mM Tris-HCl, pH 8.0 - 3 mM NaN_3, and the column was eluted by the same buffer. Fractions (4 ml) were collected every 15 min. Protein complexes were located by optical density at 280 nm (0-0). The column was calibrated as described (7).

was not further resolved. (ii) Fraction I produced only one sharp peak in isoelectric focusing in the presence of a non-ionic detergent. (iii) Amino terminal analysis produced only dansylalanine.

Heat stability of the protein aggregate. The SDS-insoluble fraction of Table I was heated in SDS. The oligomer of 35,000 dalton protein was not dissociated by heating for 5 min at temperatures up to 70°. It became partially, and completely disaggregated, by treatment at 80°, and 90°, respectively (Fig. 3). When preparations heated at various temperatures were tested for permeability-conferring activity after removal of SDS by dialysis, the extent of disaggregation was found to parallel the loss of permeability-conferring activity (Fig. 3).

Fidelity of reconstitution. I examined whether the permeability proper-

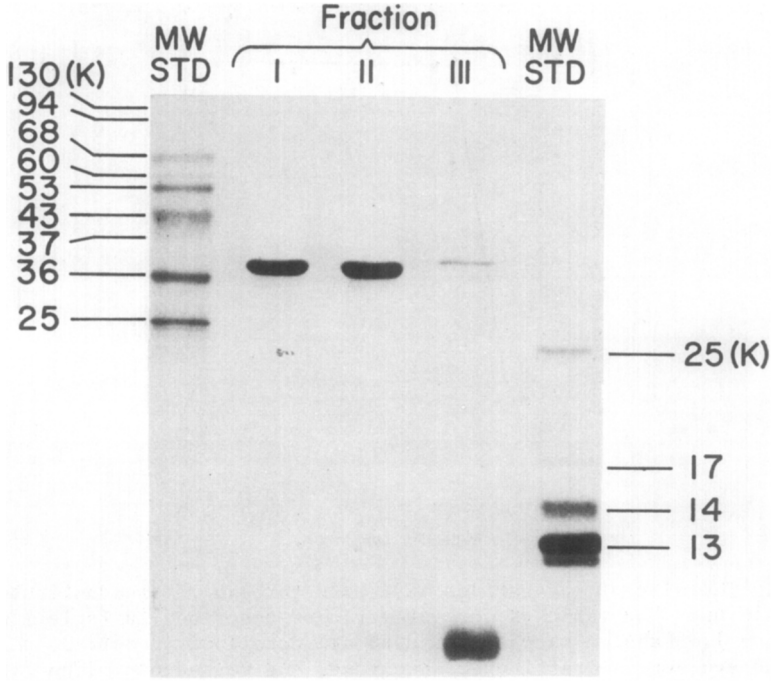

Fig. 2. Protein composition of various preparations. The samples were heated in SDS-containing "sample buffer" at 100° for 5 min., and portions each containing 3 µg protein were separated by electrophoresis in 14% acrylamide gel containing SDS (11). Molecular weight references used have been described (7).

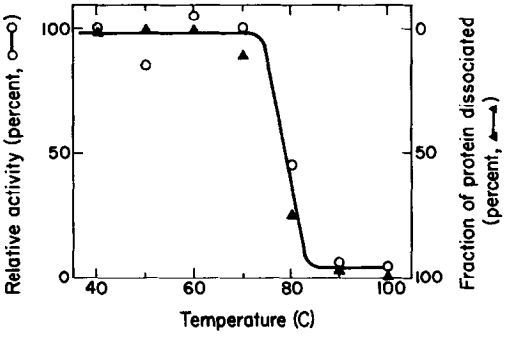

Fig. 3. Heat stability of the pore protein aggregates. The SDS-insoluble protein (500 µg) was suspended in 0.1 ml of 1% SDS in a small test tube. Tubes were heated in water bath for 5 min at desired temperature and chilled immediately in crushed ice. A portion of material was used for permeability assay after removal of SDS by dialysis, and another portion for SDS-acrylamide gel electrophoresis without further heat treatment. The vesicles were reconstituted from 1 µmole of phospholipids, 0.05 µmoles of LPS and 100 µg of heated protein, and sucrose permeability of the vesicles was determined (7). Amounts of dissociated protein was determined from densitometer reading of stained SDS-gel electrophoregram and expressed as percentage of the dissociated protein versus total protein applied on.

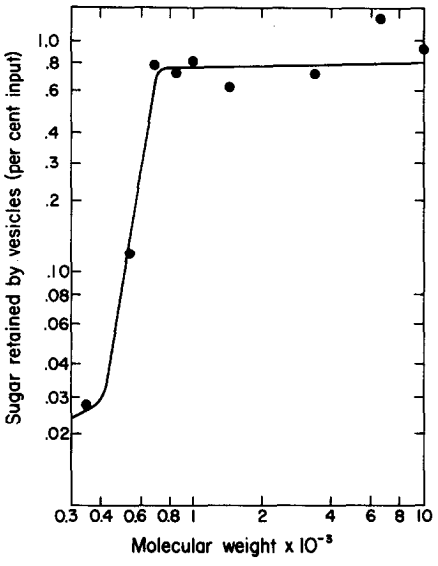

Fig. 4. The size of saccharides that pass through the reconstituted outer membrane vesicles. The vesicles were prepared as described in Table 1 with 200 µg of Fraction I. Labeled saccharides used are described in ref. 3; oligosaccharides used are sucrose raffinose, stachyose, and verbascose. The figure shows the sugar retained by vesicles, in terms of a percentage of the total input of radioactivity.

ties of the reconstituted vesicles resembled those of the native outer membrane. The reconstituted membrane (Fig. 4), as well as the intact outer membrane of E. coli B (G. M. Decad and H. Nikaido, manuscript in preparation), were permeable for sucrose and raffinose, but not for saccharides larger than stachyose. The reconstituted membrane was also permeable for UMP, fructose, mannose, galactose, lysine, leucine, aspartic acid, and polyethyleneglycol of molecular weights 400 and 600, but not to polyethyleneglycol of molecular weight 1,540.

DISCUSSION

Fraction I of Fig. I is an oligomer of only one kind of protein, which presumably corresponds to the "matrix protein" of Rosenbusch (15) [also called protein I (16) and 1 (17)] on the basis of its molecular weight, isoelectric point, and NH_2-terminal amino acid. This protein is also known to be resistant to trypsin in its oligomeric form (14). This oligomer, but not other

aggregates or other outer membrane proteins extracted with SDS, confers, to the phospholipid-LPS model membrane, permeability to a wide variety of small hydrophilic molecules. These results, together with the observation that the diffusion of [^{14}C]sucrose across the vesicle membrane is very rapid even at 0° (T. Nakae, unpublished results), suggest strongly that the small molecules diffuse through pores. It seems likely that the protein oligomer in Fraction I forms aqueous pore(s), and penetrates through the thickness of the membrane in reconstituted vesicles as well as in native outer membrane, thus conferring hydrophilic permeability to that membrane. Since proteins with a similar function are expected to be present also in other membranes, I propose to call them porins (Gr. πόρος, pore).

The comparison of the apparent size of Fraction II (about 250,000 daltons from Fig. I) and the monomer molecular weight (36,500 daltons according to Ref. 15) suggests that Fraction II may consist of hexamers or octamers. Interestingly, oligomers of porins or "matrix protein" were observed electron microscopically in SDS-treated cell envelope preparations (15); the number of these "particles" suggested that each consisted of a trimer or hexamer (18).

An observation that on first sight contradicts our hypothesis is the presence of mutants that "lack" the outer membrane proteins in 33,000-38,000 daltons region, yet can grow normally in ordinary media (19). Possibly the mutants still produce small numbers of proteins which may nevertheless be sufficient to allow the diffusion of nutrients and waste products. Alternatively, there may be other outer membrane proteins that can form pores.

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