

Lysozyme-Promoted Association of Protein I Molecules in the Outer Membrane of *Escherichia coli*

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Incubation of whole envelopes prepared from sonically oscillated *Escherichia coli* K-12 cultures with lysozyme in vitro resulted in the appearance of a protein species with an apparent molecular weight double that of outer membrane protein I. Similar dimers were also detected in purified outer membranes and whole envelopes from lysozyme-induced spheroplasts of *E. coli* K-12. This was confirmed by two-dimensional electrophoresis in which the dimers were resolved in the second dimension to run as single polypeptides of protein I. Formation of dimers was correlated with peptidoglycan degradation, but the ability of protein I molecules to associate may vary between strains of *E. coli*, since dimers were found only in outer membranes from *E. coli* W7. We suggest that extensive degradation of peptidoglycan leads to nonspecific formation of protein I aggregates, but that these aggregates do not occur in vivo.

The cell envelope of *Escherichia coli* and other gram-negative bacteria consists of three distinct layers: an inner (cytoplasmic) membrane, a peptidoglycan layer external to the inner membrane, and an outer membrane (12). Because the outer membrane constitutes a penetration barrier to many antibiotics (28) and has a role in the pathogenesis of infections caused by enteric organisms (11), many recent investigations have been directed towards elucidation of the structure of this membrane with a view to clarifying its function. Thus, the properties of outer membrane proteins, phospholipids, and lipopolysaccharides have all been dealt with extensively in recent years (4, 13, 14, 16, 17, 20-23, 26, 28, 29, 32, 34-38). However, in contrast, the role of peptidoglycan in the organization of the outer membrane has received little attention, although it has been known for some time that at least two of the major outer membrane proteins of *E. coli* are peptidoglycan associated. Outer membrane protein I (13, 16, 17), which is identical to Rosenbuch's matrix protein (35), is tightly (but noncovalently) associated with peptidoglycan (22, 35), whereas protein IV (13, 16, 17), which is identical to Braun's lipoprotein (4, 5), is covalently linked at its carboxyl terminus to peptidoglycan (4, 5).

Proteins I and IV have both been implicated in the permeability of the outer membrane to hydrophilic molecules (19, 26) and, although damage to the peptidoglycan layer alters the permeability of the outer membrane (27), it is not known whether this causes reorganization of these proteins. We show here that extensive

degradation of the peptidoglycan layer by lysozyme leads to the formation of protein I aggregates in the outer membrane.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strain JC3272 (1) was used in most experiments. *E. coli* K-12 3300 (25), a strain that forms β -galactosidase constitutively, and *E. coli* W7 (5) were used where indicated. The latter requires *meso*-diaminopimelate (Dpm; 10 μ g/ml) for growth in the medium described here.

Chemicals. [2-³H]glycerol (0.143 mCi/ μ mol), L-[4,5-³H]leucine (100 mCi/ μ mol), L-[U-¹⁴C]histidine (300 μ Ci/ μ mol), and (DL + *meso*)-2,6-diamino[G-³H]pimelic acid dihydrochloride (300 μ Ci/ μ mol) were purchased from Radiochemical Centre, Amersham, United Kingdom. Lysozyme (EC 3.2.1.17) (grade 1; crystallized three times) was obtained from Sigma Chemical Co., London. Other chemicals were the purest commercially available.

Culture medium. Bacteria were grown at 37°C in a nutrient broth consisting of Difco beef extract (0.6%, wt/vol), Difco peptone (1.0%, wt/vol), and NaCl (0.5%, wt/vol), pH 7.5.

Isolation of whole envelopes and inner and outer membranes. Whole envelopes were prepared from late-logarithmic-phase cultures (8×10^8 bacteria per ml) as described by Ball et al. (P. R. Ball, I. Chopra, and S. J. Eccles, *Biochem. Biophys. Res. Commun.*, in press). Inner and outer membranes were separated from similar cultures as described previously (40).

Labeling of peptidoglycan in *E. coli* W7. *E. coli* W7 was grown in nutrient broth supplemented with [³H]Dpm (0.1 μ Ci/ μ g; total Dpm concentration, 10 μ g/ml). The quantity of labeled Dpm incorporated into peptidoglycan in various cell fractions (see Results) was determined by estimation of cold (4°C)

trichloroacetic acid-precipitable radioactivity. Trichloroacetic acid precipitates were prepared for scintillation spectrometry as described previously (8).

Chemical analyses. Protein was determined by the method of Lowry as modified by Herbert et al. (15). Lipid phosphorus was determined as described by Chen et al. (7). 2-Keto-3-deoxyoctonate (KDO) was determined as described by Ball et al. (in press), and the KDO content was expressed in arbitrary units.

Preparation of substrate for phospholipase assay. Total phospholipid labeled with [2-³H]glycerol was prepared as follows. Strain JC3272 was grown for three to four generations in nutrient broth supplemented with [2-³H]glycerol (0.5 μ Ci/ml), and the culture was extracted directly by the method of Bligh and Dyer (3). The chloroform phase was extracted twice with benzene (33), and the lipid extract was lyophilized.

Enzyme assays. The β -galactosidase (EC 3.2.1.23) content of crude lysates and membrane preparations from strain 3300 was determined as described previously (10). All preparations were dialyzed (18 h, 4°C) against the assay buffer (50 mM potassium phosphate, pH 7.0) before determinations.

Succinic dehydrogenase (EC 1.3.99.1) was assayed by following the reduction of 2,6-dichlorophenolindophenol (DCPIP) ($\epsilon_{600} = 16,100$, pH 7.0) spectrophotometrically. Incubation mixtures contained 112.5 μ mol of potassium phosphate (pH 7.0), 10 μ mol of KCN, 0.2 μ mol of DCPIP, 0.2 μ mol of phenazine metasulfate, 85 μ mol of sodium succinate, and membrane fraction (10 to 100 μ g of protein) in a volume of 3.0 ml. The absorbance at 600 nm was determined over a 10- to 20-min period at 22°C in a Unicam SP1800 recording spectrophotometer, and the observed reaction rates were corrected by subtracting absorbance changes in control cuvettes lacking substrate.

Phospholipase (phospholipase A and lysophospholipase) (31) activity was determined, as described previously (31), by using the labeled substrate (0.09 μ Ci/ μ mol, calculated assuming an average molecular weight of 700 for phospholipids). Portions of the aqueous phase containing water-soluble products derived from the labeled phosphoglycerides were taken to dryness in scintillation vials, and radioactivity was determined as described previously (9). The observed rates of reaction were corrected for nonspecific transfer of radioactivity to the aqueous phase.

Polyacrylamide gel electrophoresis. Disc gel electrophoresis was performed as described by Weber and Osborn (39), except that membranes were solubilized directly by heating at 70°C for 20 min in 10 mM sodium phosphate (pH 7.0) containing 1% (wt/vol) dodecyl sulfate and 1% (vol/vol) β -mercaptoethanol. A 2-mg amount of protein was added per ml of denaturing buffer, and 50 μ l (100 μ g of protein) was applied to each gel. Electrophoresis was performed, as described previously (39), with 10-cm gels.

Two-dimensional separations were performed as follows. Slab gels (23) 5 mm thick were cast in a Shandon vertical polyacrylamide gel apparatus to provide a running gel (11% [wt/vol] acrylamide) of

approximately 17 cm and a stacking gel (3% [wt/vol] acrylamide) of 2 cm. Disc gels (as above) containing 200 μ g of separated membrane polypeptides were removed from their tubes, and the gels were individually equilibrated at room temperature in 100 ml of 0.0625 M tris(hydroxymethyl)amino-methane (pH 6.8) containing 2% (wt/vol) dodecyl sulfate, 10% (vol/vol) glycerol, and 5% (vol/vol) β -mercaptoethanol. The gels were then heated at 100°C for 5 min in the same solution, cooled, and applied to the upper edge of the slab separating gel. Hot agarose containing solution O (30) was pipetted above and below the disc gels to fix them in position. Electrophoresis (50-mA constant current for 16 to 18 h) was performed as described previously (23). Migration of protein from the first-dimension gels into the separating slab gels was quantitative. The slab gel system described here can resolve all of the major outer membrane proteins (23).

Polypeptides were visualized in both gel systems, and their apparent molecular weights were determined as described previously (9). For the slab system, denatured standard proteins (10 μ g; 23) were applied to wells formed in the agarose. Densitometry of disc gels was performed as described previously (9).

Radioactive labeling experiments to identify protein IV. Double-label experiments were performed by growing strain JC3272 in the presence of L-[U-¹⁴C]histidine (0.02 μ Ci/ml) and L-[4,5-³H]leucine (0.25 μ Ci/ml). Whole envelopes and outer membranes were prepared as described above, and their labeled proteins were denatured and separated by the disc gel system also described here. After electrophoresis the gels were sliced into 1-mm sections with a Mickle gel slicer. Samples were prepared for estimation of radioactivity by using tissue solubilizer (Nuclear-Chicago) followed by the addition of a Triton-toluene scintillator (8). The ³H and ¹⁴C contents of each slice were determined in a Packard Tri-Carb liquid scintillation spectrometer under conditions in which there was no overlap of ³H into the ¹⁴C channel, and the overlap of ¹⁴C activity into the ³H channel was 5.3%. The counts recorded in the ³H channel were corrected for this overlap.

Visualization of carbohydrate in disc gels. Staining of gels for carbohydrate was performed as described previously (18), using bovine serum mucin as a staining standard.

RESULTS

Formation of a protein species with an apparent molecular weight of 45,100 by incubation of *E. coli* K-12 JC3272 whole envelopes with lysozyme. Envelopes prepared from strain 3300 contained less than 0.25% of the β -galactosidase activity found in crude sonic extracts. This probably indicates negligible contamination of envelopes with cytoplasmic proteins. Analysis of the protein content of whole envelopes from strain JC3272 by disc gel electrophoresis demonstrated a number of polypeptides (Fig. 1B) with apparent molecular weights be-

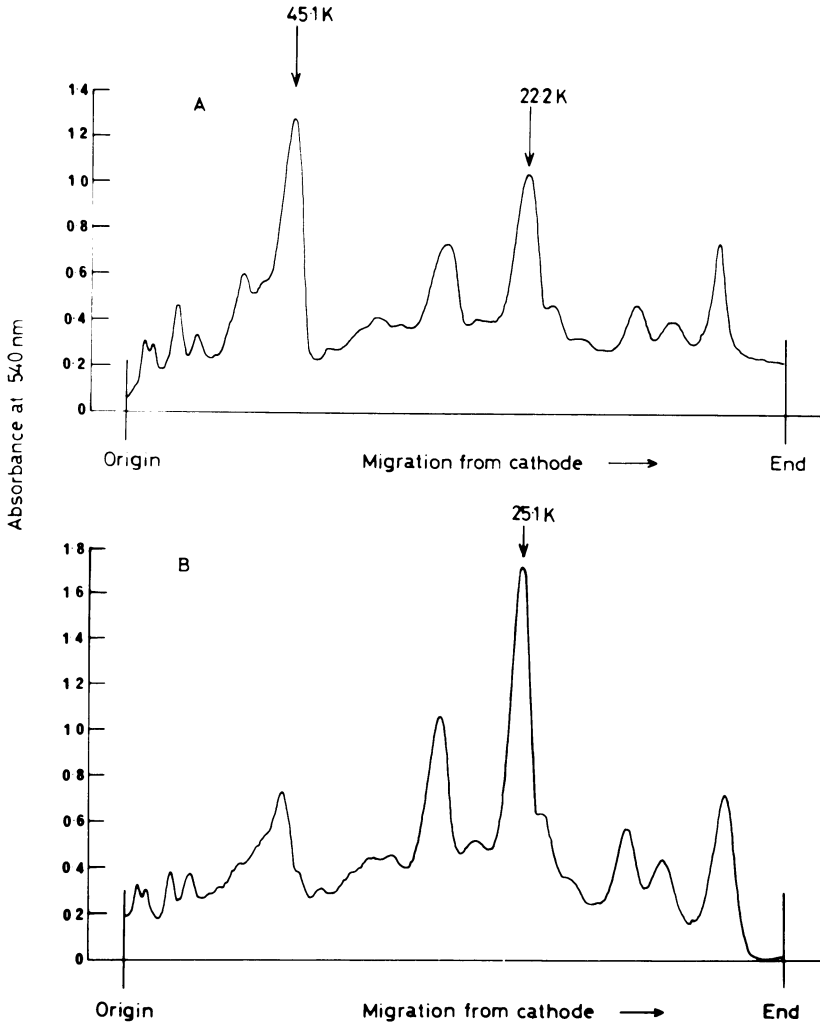


FIG. 1. Scanning densitometer tracings of disc gels containing separated proteins from envelopes of strain JC3272 treated with (A) or without (B) lysozyme. Envelopes were prepared from sonically treated cultures of JC3272, suspended at a protein concentration of 0.7 mg/ml of 10 mM sodium phosphate (pH 7.0), and incubated at 4°C for 15 min with (A) or without (B) lysozyme (50 μ g/ml). Envelopes were harvested and washed three times by centrifugation (2-h periods, $80,000 \times g$, 4°C) in 10 mM sodium phosphate (pH 7.0) and then subjected to disc gel electrophoresis as described in the text. Gels were stained with Coomassie brilliant blue, destained, and scanned in a gel densitometer at 540 nm. Proteins with apparent molecular weights of 45,100, 22,200 (1A), and 25,100 (1B) are indicated.

tween 13,000 and approximately 90,000. One of the major polypeptides with an apparent molecular weight of 25,100 (Fig. 1B) was identified as outer membrane protein I by two-dimensional electrophoresis (data not shown). Incubation (4°C) of these envelopes with lysozyme (50 μ g/ml) produced a major peak with an apparent molecular weight of 45,100 (Fig. 1A). The content of protein I, with an apparent molecular weight of 22,000 (again identified by two-dimensional electrophoresis; see Fig. 4), was reduced in lysozyme-treated preparations

(Fig. 1). These findings suggested that the higher-molecular-weight protein species may be a dimer of protein I formed as a consequence of extensive peptidoglycan degradation, but confirmation that the lysozyme treatment did indeed lead to peptidoglycan degradation was required.

The envelope polypeptide that migrated furthest towards the anode probably represented peptidoglycan-linked lipoprotein (protein IV) (20). Its identity was confirmed by experiments in which the envelope proteins were labeled

with radioactivity derived from [^{14}C]histidine and [^3H]leucine. The $^{14}\text{C}/^3\text{H}$ ratio in the peak containing protein I was 0.72, whereas in the most rapidly migrating peak it was 0.38. These values are consistent with the known amino acid compositions of proteins I (13) and IV (4, 5), the latter lacking histidine. Lysozyme treatment reduced the apparent molecular weight of the lipoprotein complex from $13,200 \pm 500$ (six determinations) to $9,500 \pm 750$ (seven determinations) ($P < 0.001$ by Student's t test). These data are most readily explained by cleavage of peptidoglycan from the lipoprotein complex and correspond (20) to the removal of three repeating disaccharide units.

To provide an alternative system for detection of peptidoglycan degradation, envelopes were prepared from *E. coli* W7 labeled with [^3H]Dpm. Since this strain can neither synthesize Dpm nor convert it to lysine (Dpm decarboxylase negative) (5), [^3H]Dpm is incorporated directly into peptidoglycan. Lysozyme treatment (Fig. 1) reduced the specific activity of W7 whole envelopes from 575 cpm/ μg of protein to 115 cpm/ μg of protein, which corresponds to the cleavage of 2.5 repeating disaccharide units of the peptidoglycan. However, in contrast to the data obtained with JC3272 envelopes (Fig. 1), lysozyme treatment of W7 whole envelopes did not produce a higher-molecular-weight protein species (data not shown).

Presence of high-molecular-weight protein species in purified outer membranes from *E. coli* K-12 JC3272 and *E. coli* W7. If extensive peptidoglycan degradation results in aggregation of protein I in the outer membrane, then it should be possible to detect such aggregates in outer membranes that have been prepared from lysozyme-induced spheroplasts. Accordingly, spheroplasts were prepared with lysozyme, and the presence of aggregates was determined in the resultant outer membranes and the envelope fractions from which they were prepared.

The purity of outer membranes was established by estimation of components (Table 1) known to be preferentially associated with either the inner or outer membranes (31, 40). Thus, outer membranes were enriched for phospholipase and KDO but contained little succinic dehydrogenase (Table 1). Contamination of membranes with cytoplasmic proteins was probably negligible on the basis of the β -galactosidase content of fractions prepared from strain 3300 (Table 1).

Analysis of the outer membrane protein content from strain JC3272 by disc gel electrophoresis demonstrated a number of protein

TABLE 1. Location of components upon fractionation of strain 3300 by the method of Yamato et al. (40)^a

Component	Activity or content in:		
	Crude lysate	Outer membrane	Inner membrane
β -Galactosidase	400.8	1.4	2.6
Succinic dehydrogenase	ND ^b	0.2	45.0
Phospholipase	ND	14.0	2.6
KDO	ND	30.5	13.5

^a The crude lysate obtained by sonic treatment of spheroplasts (40) was fractionated (40) to produce inner and outer membranes. Components were determined as described in the text. Enzyme activities are expressed as nanomoles per minute per milligram of protein, except for phospholipase (picomoles per minute per milligram of protein). Values for enzyme activities are the means of three separate determinations for each fraction. The KDO content is expressed as arbitrary units per milligram of protein. Values given are the means of four separate determinations for each membrane.

^b ND, Not determined.

peaks (Fig. 2). Subsequent two-dimensional electrophoresis (e.g., see Fig. 4) suggested that peaks B and C comprised protein I and peak D of Henning's protein II* (16), respectively. Only peak E (apparent molecular weight, 18,000) gave a positive reaction when the gels were stained for carbohydrate (data not shown) and, therefore, probably represented Henning's protein III, which is often contaminated with lipopolysaccharide (17). Peak F was the peptidoglycan-linked form of lipoprotein, since this peak had a $^{14}\text{C}/^3\text{H}$ ratio of 0.23 in a dual-label experiment with [^{14}C]histidine and [^3H]leucine. In contrast, the ratios were 0.65 for peak A, 0.68 for peak B, and 0.73 for peak C.

The apparent molecular weight of peak A was 61,600, and that of peak C was 29,900 (Table 2), the latter containing one of the protein I species detected (see Fig. 4). The apparent molecular weight of peak F (peptidoglycan-linked lipoprotein) in the outer membrane preparations was $9,200 \pm 800$ (five determinations), which indicated a degree of peptidoglycan degradation similar to that in the envelopes treated with lysozyme in vitro. Denaturation of outer membranes for disc gel electrophoresis by heating at 100°C for 5 min (as opposed to 70°C for 20 min) caused peak A to disappear and to be replaced by a single, broad peak whose mobility was similar to that of peak C (data not shown).

These data for the outer membrane of strain JC3272 are consistent, therefore, with the view that extensive peptidoglycan degradation causes aggregation of protein I to form noncovalently linked dimers. The possibility that peak

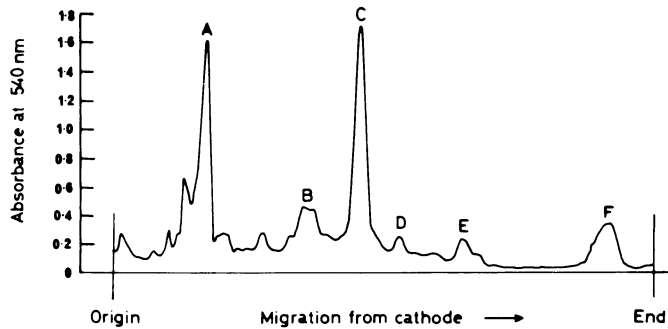


FIG. 2. Scanning densitometer tracing of a disc gel containing separated outer membrane proteins from strain JC3272. Details of electrophoresis as for Fig. 1. The peaks identified by letters correspond to proteins with apparent molecular weights as follows: A, 61,000; B, 37,900; C, 29,900; D, 25,100; E, 18,000; F, 9,200.

TABLE 2. Apparent molecular weights of protein I and the aggregates in various preparations^a

Prepn	Apparent molecular weight of:	
	Protein I ^b	Aggregate
Lysozyme-treated envelopes	22,200 ± 700 (7)	45,100 ± 1,100 (7)
Envelopes from sonically treated bacteria	25,100 ± 1,100 (6)	Not present
Outer membranes	29,900 ± 2,200 (5)	61,600 ± 2,700 (5)
	37,900 ± 2,100 (5)	
Envelopes from lysozyme-induced spheroplasts	23,400 ± 1,800 (2)	50,800 ± 4,100 (2)

^a Preparations were analyzed by disc gel electrophoresis, and the apparent molecular weights of proteins were determined as described in the text. Values given are ± standard deviation, with the number of determinations in parentheses. Treatment of envelopes with lysozyme was performed as described in the legend to Fig. 1A. Protein I was identified by two-dimensional electrophoresis (see text).

^b Two forms of protein I were detected in outer membranes (see text).

A formed during separation of outer from inner membranes was assessed by analysis of JC3272 whole envelopes prepared by the scheme (40) adopted for the separation of the two membranes. A major peak (arrow, Fig. 3) was found in the region of the gels containing high-molecular-weight polypeptides. This material, with an apparent molecular weight of 50,800, again appeared to be an aggregate of protein I (Table 2).

Protein species with molecular weights twice those of protein I were not detected in envelopes of *E. coli* W7 (prepared from lysozyme-induced spheroplasts) but were found in outer membranes from this strain (data not shown). Lysozyme treatment of labeled (³H]Dpm) W7 whole cells to produce envelopes caused about a threefold reduction in the specific activity of [³H]Dpm (in relation to KDO content) (Table 3). The specific activity was greatly reduced in the enriched outer membrane fraction (Table 3), indicating considerable cleavage and removal of peptidoglycan during outer membrane preparation.

Identification of protein aggregates as dimers of protein I. Although the apparent molecular weight of protein I and its putative

aggregate varied, the apparent molecular weight of the aggregate was always approximately twice that of protein I (Table 2). This was confirmed by the two-dimensional separation of proteins from lysozyme-treated whole envelopes (Fig. 4A) and outer membranes (Fig. 4B) of strain JC3272, where the second dimension resolved the major outer membrane proteins (23). The protein aggregates separated in the first-dimension (disc) gels were degraded to form products (spots 1 and 3, Fig. 4) with apparent molecular weights of 38,000. Proteins in spots 2, 4, and 5 (Fig. 4) were derived, respectively, from the peak with an apparent molecular weight of 22,200 in the whole envelopes and from peaks B and C in the outer membranes. Since protein I has an apparent molecular weight of 38,500 in this slab gel system (23), spots 1 through 5 all appear to represent protein I. Peak B (Fig. 2), derived from outer membranes, probably represents a peptidoglycan-protein I complex (35) that disappeared upon heating the first-dimension gels at 100°C.

DISCUSSION

With improved gel electrophoresis systems (23), protein I of *E. coli* K-12 can now be

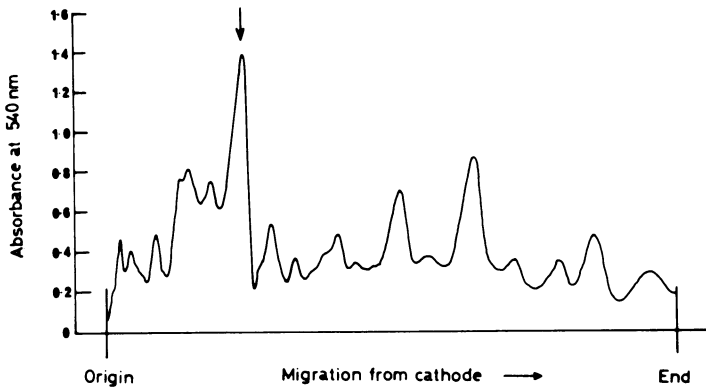


FIG. 3. Scanning densitometer tracing of a disc gel containing separated proteins from envelopes prepared from lysozyme-induced spheroplasts of strain JC3272. Whole envelopes were prepared as described previously (40) and then subjected to electrophoresis as described in the legend to Fig. 1. The arrow indicates polypeptides of apparent molecular weight 50,800 (see text).

TABLE 3. Specific activity of [^3H]Dpm upon fractionation of strain W7 by the method of Yamato *et al.* (40)^a

Fraction	[^3H]Dpm sp act (cpm/KDO unit)
Washed whole cells	98,633
Whole envelopes	29,242
Inner membranes	1,027
Outer membranes	4,286

^a Strain W7 was labeled with [^3H]Dpm (0.1 $\mu\text{Ci}/\mu\text{g}$) as described in the text. Bacteria were harvested by centrifugation (10,000 $\times g$, 4°C, 20 min) and washed three times with 30 mM tris(hydroxymethyl)aminomethane (pH 8.0) containing 100 μg of nonradioactive Dpm per ml. The washed bacteria were fractionated to produce whole envelopes and inner and outer membranes as described previously (40). The quantity of labeled Dpm in the peptidoglycan and the KDO content of each fraction were determined as described in the text. The data are calculated from at least duplicate determinations of both radioactivity and KDO for each fraction.

resolved into two protein bands, designated I_a and I_b (17). Although we used the improved gel technique (23) in part of our studies, we were unable to resolve two protein I components. This may be attributed to the manner in which we grew the organisms, since the nature of the growth medium greatly influences the content of these proteins in the membrane (24).

Nakae (26) concluded from his studies on liposomes that aggregates of protein I exist in the outer membrane to produce transmembrane pores through which small hydrophilic molecules diffuse. Although such pore-forming proteins would be expected to span at least the lipid bilayer of the outer membrane, the evidence on this point is equivocal. Thus, protein

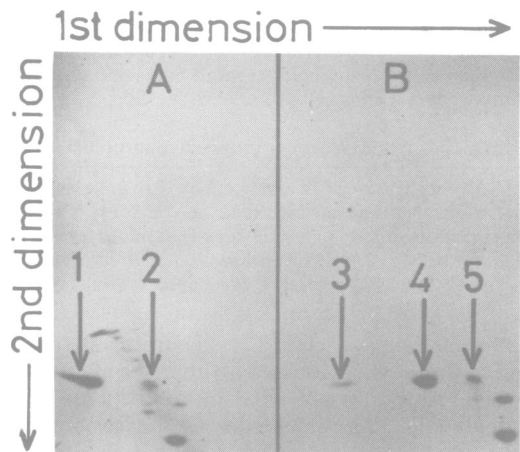


FIG. 4. Two-dimensional separation of proteins from (A) lysozyme-treated envelopes and (B) outer membranes of strain JC3272. Envelopes were prepared, treated with lysozyme, and washed three times as described in the legend to Fig. 1A. Outer membranes were prepared as described in the text. These preparations were then subjected to two-dimensional electrophoresis as described in the text. Spots 1 through 5 contain protein with an apparent molecular weight of 38,000 (see text).

I (the matrix protein) exists primarily as a β -structured polypeptide in close (but noncovalent) association with the outer face of the peptidoglycan (35), so that little of the polypeptide chain may actually penetrate the bilayer of the outer membrane. This is also consistent with the absence of long sequences of hydrophobic amino acids in this protein (13). On the other hand, protein I_a interacts with the heptose-bound phosphate group of lipopolysaccharide (24), that is assumed to be present near the surface of the cell, and protein I_b may

be a phage receptor (37).

Despite the inability to conclude that protein I traverses the membrane bilayer, two studies do support its role in the formation of hydrophilic channels. Beacham et al. (2) showed that *E. coli* K-12 mutants cryptic for the expression of periplasmic nucleotidases lacked an outer membrane protein (probably protein I) and concluded that this caused the outer membrane to become less permeable to the substrates of the periplasmic enzymes. Nikaido et al. (29) showed that *Salmonella typhimurium* mutants deficient in major outer membrane proteins (possibly similar to the *E. coli* proteins I_a and I_b) were impermeable to cephaloridine compared with wild-type bacteria.

To establish conclusively that aggregates of protein I themselves form transmembrane channels (as suggested by Nakae), it would seem necessary to demonstrate the protein complexes in vivo. Although neighboring molecules of protein I are probably only separated by 1.3 nm in the outer membrane of *E. coli* K-12 (32), Palva and Randall's data (32) suggest that aggregates of protein I probably do not occur in vivo. In view of these findings and the data presented in this paper, we suggest that peptidoglycan maintains the separation of neighboring protein I molecules in the envelope and that its degradation permits protein I to aggregate. Where protein I was associated with peptidoglycan (peak B, Fig. 2; Fig. 4), the content of protein I aggregates in the outer membrane (peak A, Fig. 2) was reduced, supporting the view that peptidoglycan controls the ability of neighboring protein I molecules to associate.

The extent of peptidoglycan degradation required before protein I will associate may vary between strains of *E. coli*. Thus, treatment of envelopes from the K-12 strain JC3272 with lysozyme in vitro caused aggregate formation, but similar treatment of W7 envelopes failed to produce protein dimers even though the extent of peptidoglycan degradation was similar in both cases. Since dimers of protein I in W7 were only detected in enriched outer membranes, it seems that more extensive peptidoglycan degradation is required for the protein molecules to associate in this strain than in JC3272.

The exact manner by which protein I contributes to the formation of diffusion channels is therefore unclear, but we believe that its ability to form aggregates may be artificial. In this context it is interesting that another major outer membrane protein, protein IV (Braun's lipoprotein), tends to form aggregates when

the isolated protein is suspended in aqueous solutions (6, 21).

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