Proteins Released from Cell Envelopes of *Pseudomonas aeruginosa* on Exposure to Ethylenediaminetetraacetate: Comparison with Dimethylformamide-Extractable Proteins

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Isolated cell envelopes of *Pseudomonas aeruginosa* were treated with N,N'-dimethylformamide (DMF) or with ethylenediaminetetraacetate (EDTA). DMF solubilized 73% of the dry weight of the cell envelope, 76% of the protein, 78% of the carbohydrate, and 76% of the phosphorus. Electron microscopy showed that DMF caused extensive alterations in the appearance of the cell envelope with blebs and bleblike vesicles predominating. After incubation with EDTA, the cell envelopes appeared to have lost material, but still retained the cell-like morphology. Analysis of DMF-solubilized proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed 16 protein bands. There were three major proteins that predominated, however, with molecular masses of 43,000 (protein A), 16,500 (protein B), and 72,000 daltons (protein C). Evidence is presented that protein A and protein B are glycoproteins. Gel electrophoresis of EDTA-solubilized material revealed that a number of proteins were released from the cell envelope. However, electrophoresis of an isolated protein-lipopolysaccharide complex released by EDTA showed that protein A and protein B were the major protein components of this complex. These data suggest that protein A and protein B are components of the outer cell wall membrane of *P. aeruginosa*. There is suggestive evidence that these proteins may play a role in maintaining the structural integrity of the cell envelope. Whether these proteins also have enzymatic activity could not be discerned from the present study, although it is possible that they may be associated with the terminal stages of lipopolysaccharide synthesis.

Gram-negative bacteria characteristically have a cell envelope consisting of two membranes separated by a thin peptidoglycan layer. *Pseudomonas aeruginosa* is typical in this respect, but is atypical in that it is lysed (11, 14, 26) by the action of ethylenediaminetetraacetate (EDTA). The lethal action of EDTA has been attributed to the chelation of divalent metals which are required for the structural integrity of the envelope (1, 2, 10, 11, 27). Rogers, Gilleland, and Eagon (28) demonstrated that a protein-lipopolysaccharide (PrLPS) complex is released from the cell envelope by EDTA. This complex was further purified by Roberts, Gray, and Wilkinson (27) and found to be 60% protein, 30% lipopolysaccharide, and 10% loosely bound lipid.

In order to elucidate the structure of the envelope of *P. aeruginosa*, and to pinpoint the site of action of EDTA, studies have centered around the chemical composition of the envelope with respect to lipid and lipopolysaccharide (5, 9, 10, 12, 27, 28). However, the major component of the envelope, the protein, has been neglected thus far. Before the gram-negative cell envelope can be adequately understood, it is necessary that the protein components be described fully and that their function in ultrastructure be determined.

Schnaitman (31) reported a technique for analyzing *Escherichia coli* cell envelope proteins by using acidified N,N'-dimethylformamide (DMF) extraction to prepare lipid-free protein, which was followed by separation of
the proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In continuing our investigation into the cell envelope chemistry and ultrastructure of _P. aeruginosa_, this report describes an analysis of the protein components in the cell envelope and those specific proteins released by EDTA by using techniques of DMF extraction and polyacrylamide gel electrophoresis to isolate and separate proteins.

**MATERIALS AND METHODS**

**Cultivation of organism.** _P. aeruginosa_ strain OSU 64 was maintained on tryptic soy agar slants (Difco Laboratories, Detroit, Mich.) by weekly transfers. Cells to be used in the preparation of envelopes were grown by inoculating a 500-ml Erlenmeyer flask containing 100 ml of tryptic soy broth (TSB) (Difco) with 0.1 ml of a suspension prepared by washing a slant of 24-h growth with 1 ml of TSB. The flask was incubated for 5 h at 30°C on a rotary shaker, and then 0.2 ml was used to inoculate 1-liter Erlenmeyer flasks, each containing 200 ml of TSB. These flasks were then incubated for 14 h at 30°C on a rotary shaker.

**Preparation of cell envelopes.** Fourteen-hour broth cultures were harvested, and cell envelopes were prepared as previously described (5), with the modification that the envelopes were washed only once with 0.1 M phosphate buffer, pH 5.5, and then were washed twice with deionized water.

**Preparation of EDTA-soluble cell envelope material.** Isolated cell envelopes were treated with EDTA in tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.0, as previously described (28). The 37,000 × _g_ supernatant fluid, termed "EDTA-soluble," was found by electron microscopy to contain the PrLPS complex, as previously described (28), along with unidentified membrane vesicles. This material was fractionated by centrifugation at 100,000 × _g_ for 1 h at 4°C in 35 mM Tris-hydrochloride, pH 8, and 1 mM EDTA to give a "100,000 × _g_ pellet" and a "100,000 × _g_ supernatant fraction." Electron microscopy showed the PrLPS complex in the 100,000 × _g_ supernatant fraction.

The complex was further purified by gel filtration by using Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) as follows. The 100,000 × _g_ supernatant fraction of EDTA-soluble material from 100 mg of cell envelopes was dialyzed for 24 h at 4°C against 33 mM Tris-hydrochloride (pH 8.0) plus 3 mM EDTA, and then was concentrated to a volume of 5 ml by ultrafiltration (Diaflow PM-10 membrane, Amicon Corp., Lexington, Mass.). This material then was applied to the column (2.5 × 35 cm) and eluted with 10 mM Tris-hydrochloride, pH 8.0, at 25°C. The flow rate was 36 ml/h. Fractions were collected at 5-min intervals, and elution was monitored at 280 nm. The PrLPS complex was shown by electron microscopy to elute in the void volume and immediately after the void volume. Fractions containing the PrLPS complex were pooled, mixed with an equal volume of 6 mM EDTA, pH 8, and 10 mM Tris-hydrochloride (pH 8.0), and filtered through an XM-300 membrane filter (Amicon Corp., Lexington, Mass.). The filtrate was then dialyzed, for 24 h at 4°C against deionized water, and lyophilized.

**Preparation of lipid-free protein.** Proteins were solubilized and prepared for electrophoresis as described by Schnaitman (31) by using DMF (spectrophotometric grade, Mallinkrodt Chemical Works, St. Louis, Mo.) and 0.12 N HCl (9:1, vol/vol). A Sephadex LH-20 column (2.5 × 35 cm) was used to free the protein solutions of lipid. The preparations were concentrated to 1 to 2 mg of protein per ml by ultrafiltration (Diaflow PM-10 membrane, Amicon Corp., Lexington, Mass.) before electrophoresis.

**Chemical analyses.** Total protein was estimated by the biuret method (20) or by the method of Lowry et al. (24), and bovine serum albumin was used as the standard. Carbohydrate was determined by the anthrone method (3) by using glucose as the standard. Total phosphorus of acid-hydrolyzed samples was determined by the method of Chen, Toribara, and Warner (4).

**Polyacrylamide gel electrophoresis.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis and molecular weight estimations were carried out by the procedure of Weber and Osborn (34), which was modified by preparing the gels and buffer system in 1 M urea and by carrying out electrophoresis at 5 mA per gel. Protein standards for molecular weight determinations were cytochrome c, trypsin, pepsin, and glutamic dehydrogenase (all from Sigma Chemical Co., St. Louis, Mo.). Gels were stained for protein with Coomassie Blue according to Weber and Osborn (34) or for carbohydrates and glycoproteins by the method of Holden et al. (15).

**Electron microscopy.** Preparations were studied by the negative staining technique with 2% phosphotungstic acid (PTA), pH 6.1. For lyophilized preparations, a small amount of the lyophilized material was reconstituted in a drop of deionized water, and then was mixed with a drop of PTA. When non-lyophilized preparations were used, a drop of the sample was mixed directly with a drop of PTA. Some of the sample PTA mixture was then picked up on a nitrocellulose-coated 300-mesh copper grid, the excess fluid was drawn off with filter paper, and the grid was allowed to air-dry. The preparation was then observed in a Phillips 200 electron microscope.

**RESULTS**

**Cell envelopes.** No intact cells were detected in the cell envelope preparations that were used in these studies. Electron microscopy examination of these preparations showed both relatively intact cell envelopes and fragments of cell envelopes, both having a smooth, close-knit appearance (Fig. 1).

**DMF-treated cell envelopes.** DMF extraction removed 73% of the dry weight of the lyophilized cell envelopes while 76% of the protein, 78% of the carbohydrate, and 76% of the phosphorus were removed. Experiments
were not done to determine the amount of lipids and of other inorganic substances that were removed.

After extraction of cell envelopes with DMF, electron microscopy of the residual cell envelope material revealed envelope fragments with numerous blebs and with many bleb-like vesicles free in the surrounding areas (Fig. 2).

**Gel electrophoresis of proteins extracted from cell envelopes by DMF.** A large number of proteins were extracted from cell envelopes by DMF as revealed by gel electrophoresis (Fig. 3, gel A). A distinctive feature is that there are three major proteins in the preparation. Estimation by gel electrophoresis showed that these major proteins had molecular weights of 43,000 and 16,500, and 72,000. These were termed protein A, protein B, and protein C, respectively. Moreover, when the gels were stained for carbohydrate as well as for protein, carbohydrate bands corresponded with protein A and protein B, suggesting that these are glycoproteins (Fig. 4).

The range of molecular weights of the proteins that were extracted from cell envelopes by DMF was estimated to be from 5,200 to 260,000. However, the estimated molecular weights that were outside the range of standards that were used (i.e., 11,700 to 53,000) were probably not entirely accurate. The very high-molecular-weight protein bands (i.e., greater than 100,000) may be denatured protein aggregates.

**EDTA-treated cell envelopes.** The close-knit mesh appearance of normal cells envelopes was altered to give a more loosely packed appearance when the cell envelopes were incubated with EDTA (Fig. 5). This appears to result from the extraction of materials from the surface layers of the cell envelope by EDTA.

**EDTA-solubilized material from cell envelopes.** The elution profile of the 100,000 × g supernatant fraction from EDTA-treated cell envelopes when chromatographed on a Sephrose 4B column is shown in Fig. 6. The bulk of the protein was in the void volume and immediately after the void volume.

Electron microscopy of the various fractions showed that the PrLPS complex was present in the initial fractions, in which also was found the bulk of the protein. The PrLPS complex was not detected in any of the other fractions.

Electron microscopy of a negatively stained preparation of the purified PrLPS complex showed the presence of rodlets approximately 7 × 20 to 25 nm (Fig. 7). The rodlets appeared to be made up of three spherical units 7 ± 1 nm in diameter.

**Gel electrophoresis of proteins extracted from cell envelopes by EDTA.** Lyophilized samples of the 100,000 × g supernatant fluid from EDTA-treated cell envelopes and of the PrLPS complex were extracted with DMF and prepared for electrophoresis in the same manner as for intact cell envelopes. Scans of the electrophoresis gels are shown in Fig. 3. For comparative purposes, a scan of a gel of DMF-extractable proteins from cell envelopes is also shown (Fig. 3, gel A). These data show that EDTA released a number of proteins from cell envelopes as evidenced by the number of bands resulting from gel electrophoresis of the 100,000 × g EDTA supernatant fluid (Fig. 3, gel B). The PrLPS complex, however, did not contain such a wide variety of proteins (Fig. 3, gel C). In fact, when gels A and C (Fig. 3) are compared, the major proteins in the PrLPS complex appear to be protein A and protein B.

**DISCUSSION**

The total protein content of the cell envelope of *P. aeruginosa* and those envelope proteins released by EDTA were studied by using DMF to prepare lipid-free protein according to Schnaitman (31) and sodium dodecyl sulfategel electrophoresis as an analytical tool. In the course of this study, the effects of these agents on the ultrastructure of isolated envelopes were also investigated by using electron microscopy.

The DMF solubilization technique used in this study is not selective for cytoplasmic or outer-membrane proteins. From the analysis of the DMF insoluble envelopes, it is apparent that this treatment does not remove all the protein (only 76%). Any protein covalently bound to the peptidoglycan, for example, would be included in this insoluble portion.

Recently it has been shown (25) that *P. aeruginosa* does possess peptidoglycan-bound lipoprotein similar to *E. coli* (6, 7), but in lesser amounts. This lipoprotein apparently extends outward from the murein and provides a backbone and support for the outer membrane (6, 7). It is of interest that, although EDTA removes lipopolysaccharide from *E. coli*, this organism is not lysed (21, 22). The reduced amount of peptidoglycan-bound lipoprotein in *P. aeruginosa* is indicative of a basic difference between these two bacteria and may also point to the reason for the extreme EDTA sensitivity of *P. aeruginosa*.

Isolated cell envelopes treated with DMF were shown to differ ultrastructurally from untreated envelopes. The surface still appears close-knit and smooth, but numerous blebs
FIG. 1. Electron micrograph of a negatively stained (phosphotungstic acid) preparation of cell envelopes. Note the smooth close-knit appearance of the cell envelopes. The preparation consisted of large fragments of cell envelopes. The marker in the micrograph and in all subsequent micrographs represents 200 nm.

FIG. 2. Electron micrograph of a negatively stained (phosphotungstic acid) preparation of cell envelopes after extraction with N,N'-dimethylformamide. Note the extensive bleb formation which results in the loss of the cell-like morphology that was seen in the untreated cell envelope preparations (compare with Fig. 1).
extractable proteins of \textit{P. aeruginosa}

Fig. 3. Densitometric tracing of electrophoresis gels to which 100 \textmu g of protein was applied; all gels were stained with Coomassie Blue. Gel A, proteins extracted from cell envelopes with \textit{N,N’-dimethylformamide (DMF)}; gel B, proteins extracted with DMF from the 100,000 \times g supernatant fraction of the material solubilized from cell envelopes by EDTA; and gel C, proteins extracted from the protein-lipopolsaccharide (PrLPS) complex by DMF. Protein A (43,000 daltons), protein B (16,500 daltons), and protein C (72,000 daltons) are identified in gel A. This figure shows that protein A and protein B are components of the PrLPS complex (gel C). A wide variety of proteins was released from the cell envelope by EDTA (gel B). The tops of the gels are to the right. The tracing was done with a Gilford scanner at 620 nm.

Fig. 4. Diagrammatic representation of gel electrophoresis patterns in two gels stained for comparative purposes (i) with Coomassie Blue to reveal protein (left) and (ii) with the periodate-Schiff base reaction to reveal carbohydrate (right). Protein A, protein B, and protein C are indicated in the gel on the left. Note that protein A and protein B bands correspond with the carbohydrate bands in the gel on the right.

The appearance of EDTA-treated envelopes is in marked contrast to DMF-treated envelopes. These former envelopes have lost their smooth, close-knit appearance, but retain their cell-like morphology. It has been previously shown that EDTA solubilizes 30\% of the dry weight of \textit{P. aeruginosa} cell envelopes, along with 23\% of the protein, 35\% of the carbohydrate, and 5\% of the lipid (10). EDTA is not a solvent as is DMF, but it is considered to alter the cell envelope by removing divalent metals which are thought to be necessary for the structural integrity of the envelope (1, 2, 10, 11, 27).

The cell envelope of \textit{P. aeruginosa} was shown to contain a wide variety of proteins. This is consistent with reports for other gram-negative bacteria (33) and for biological membranes in general (19, 29, 30). Our results with \textit{P. aeruginosa} are similar to those reported by Schnaitman (33) in a survey of gram-negative bacteria.

Because glycoproteins have been shown to be
Fig. 5. Electron micrograph of a negatively stained (phosphotungstic acid) preparation of cell envelopes after treatment with EDTA. Note the gaps in the envelope structure and the loss of the smooth close-knit appearance (compare with Fig. 1). The cell-like morphology, however, was retained.

Fig. 6. Elution profile of the 100,000 x g supernatant fraction of material solubilized from cell envelopes by incubation with EDTA and chromatographed on Sepharose 4B. The protein-lipopolysaccharide complex was detected in fractions 10 to 23, which were in the void volume and immediately after the void volume.

common constituents of other membranes (19), we felt it was likely that some of the envelope proteins of P. aeruginosa are also glycoproteins. Indeed, analysis of the eluant of DMF-soluble proteins chromatographed on Sephadex LH-20 revealed that carbohydrate also eluted in the void column along with the protein. When electrophoresis gels were stained for carbohydrate and compared with gels stained for protein, it appeared that two of the major proteins, designated herein as protein A and protein B, are glycoproteins. It is not yet known whether the major cell envelope protein of E. coli, or of any of the other organisms surveyed by Schnaitman (33), is a glycoprotein.

We have used gel electrophoresis to show that EDTA removes a number of proteins from the cell envelope. However, studies with isolated PrLPS complex have shown that the major proteins of this complex are protein A and protein B.

The PrLPS complex resembles the endotoxin isolated from an autolysate of P. aeruginosa by Homma and co-workers (16, 17). The protein from this endotoxin was reported to have a molecular weight of approximately 42,000. It seems likely that our protein A (43,000 daltons) may be the same as this endotoxin protein reported by Homma and his associates (16, 17).

At the moment, it is impossible to assign a role to any of the proteins described herein. Proteins have been recognized as integral components of all biological membranes, but their
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Fig. 7. Electron micrograph of negatively stained (phosphotungstic acid) protein-lipopolysaccharide complex. Rodlets approximately 7 nm x 20 to 25 nm are clearly evident. Many of the rodlets are revealed to be composed of spherical units (arrows).

The striking feature of the envelopes of gram-negative bacteria studied thus far is the presence of only one or two major proteins (33). We have found three major proteins in the envelope of *P. aeruginosa*, two of which appear to be glycoproteins and are associated with the PrLPS complex which is released from the envelope by EDTA. Since lipopolysaccharide is a component of the outer membrane, it follows that protein A (43,000 daltons) and protein B (16,500 daltons) reside primarily in that locale also. However, the nature of their association with the lipopolysaccharide in the PrLPS complex is speculatory. In any case, the physical relationship seems to be hydrophobic or polar, or both, as opposed to covalent bonding (27).

We have found evidence (13) that the PrLPS complex is indeed located in the outer membrane and, moreover, that the complex may be involved in the maintenance of the structural integrity of the cell envelope. This suggests that protein A and protein B may play an important role in the cell envelope structure of *P. aeruginosa*.

Levy and Leive (23) reported that a uridine 5'-diphosphate-galactose-lipopolysaccharide (LPS) galactosyl-transferase complex active in
LPS synthesis was released from E. coli by EDTA. The site of this complex in the intact cell envelope of E. coli was not determined in their study. It is likely that the final stage of synthesis of LPS occurs in the outer cell wall membrane of gram-negative bacteria; thus, the PrLPS complex shown to be released from the outer cell wall membrane of P. aeruginosa by EDTA (13), and more fully characterized herein, may be a ternary complex of enzymes, phospholipids, and LPS involved in the terminal stages of LPS synthesis. The exact physiological role of the PrLPS complex released by EDTA from cell envelopes of P. aeruginosa, however, cannot be discerned from these present studies but will have to await the results of further experimentation.

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LITERATURE CITED


