

Outer Membrane of *Pseudomonas aeruginosa*: Heat- and 2-Mercaptoethanol-Modifiable Proteins

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A number of polyacrylamide gel systems and solubilization procedures were studied to define the number and nature of "major" polypeptide bands in the outer membrane of *Pseudomonas aeruginosa*. It was shown that five of the eight major outer membrane proteins were "heat modifiable" in that their mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis was determined by the solubilization temperature. Four of these heat-modifiable proteins had characteristics similar to protein II* of the *Escherichia coli* outer membrane. Addition of lipopolysaccharide subsequent to solubilization caused reversal of the heat modification. The other heat-modifiable protein, the porin protein F, was unusually stable to sodium dodecyl sulfate. Long periods of boiling in sodium dodecyl sulfate were required to cause conversion to the heat-modified form. This was demonstrated both with outer membrane-associated and purified lipopolysaccharide-depleted protein F. Furthermore, lipopolysaccharide treatment had no effect on the mobility of heat-modified protein F. Thus it is concluded that protein F represents a new class of heat-modifiable protein. It was further demonstrated that the electrophoretic mobility of protein F was modified by 2-mercaptoethanol and that the 2-mercaptoethanol and heat modification of mobility were independent of one another. The optimal conditions for the examination of the outer membrane proteins of *P. aeruginosa* by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis are discussed.

The outer membranes of certain gram-negative bacteria contain major polypeptides which exhibit unusual properties when examined by the technique of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (1, 3, 24). Unlike most proteins studied by this technique, the migration of these major polypeptides is determined both by the conditions of electrophoresis and by the specific manner in which the proteins are solubilized. This has led to considerable confusion in the past 5 years, especially in the case of the *Escherichia coli* outer membrane (6). A number of authors have demonstrated that major outer membrane proteins with apparent molecular weights ranging from 30,000 to 40,000 are characteristically "heat modifiable"; that is, when the outer membrane preparation is heated above a given temperature in SDS solution, the positions and number of protein bands on SDS-gel electrophoretograms are altered (1, 3, 6, 12, 20, 21, 24, 27).

The isolation of substantially purified *Pseudomonas aeruginosa* outer membranes was reported only recently by five groups (2, 10, 16, 18, 29); prior to this, hypersensitivity of *P. aerugi-*

nosa outer membranes to EDTA (16) (which was used extensively in the preparation of enteric outer membranes) created a major obstacle. Unfortunately, these recent isolations have led to five proposed schemes of nomenclature for *P. aeruginosa* outer membrane polypeptides. We have compared the various SDS-polyacrylamide gel systems used and determined how the polypeptide bands correspond to one another (Table 1). In this paper, the scheme of Mizuno and Kageyama (18) has been adopted, modifying it in two places where our 14% gel system (see below) showed two distinct polypeptide bands compared to the single one seen on 11% and 8% SDS-urea gels.

Mizuno and Kageyama (18) reported that protein F was converted to a higher apparent molecular weight form (protein F*) by either boiling for substantial periods in SDS or by trichloroacetic acid (CCl₃COOH) pretreatment. We demonstrated a similar shift (to the 37,000-molecular-weight [37K] protein) after CCl₃COOH or phenol pretreatment of outer membranes (10). However, both of the above papers suggested that protein F might consist of more than one

TABLE 1. Nomenclature of *P. aeruginosa* outer membrane proteins

Name used in this paper	Mol wt ^a (10 ³)							
	Mizuno and Kageyama	Hancock and Nikaido			Matsushita et al.		Booth and Curtiss	Stinnett and Eagon
	8% Urea	11% A ^b	11% B ^c	14%	8% Urea	10%	11%	9%
D1				46				
	D (50)		49		4(53)	4(49)	I (56)	
D2				45.5				
E	E (45)	44	45	44	4 (49)		II (53)	
F (= porin)	F (33)	35	37	39	5 (34)	5 (31)	III (38)	A (43)
G	G (21)	21	22.5	25	6 (23)	6 (20)	IV (21)	
H1				21				
	H (21)	17	18.7		7 (19)	7 (18)	V (16)	B (16.5)
H2				20.5				
I (= lipoprotein)	I (8)			9-12	9 (9.7)	9 (8.7)		

^a See references 2, 10, 16, 18, and 29 and this paper for a full description of the groups of researchers and the methods they used.

^b Using impure SDS; see reference 10.

^c Using ultrapure SDS; see the text.

polypeptide chain since under certain solubilization and electrophoresis conditions two to three bands were observed. Protein F has been partially purified and functionally characterized as a porin protein (9), which creates size-dependent transmembrane pores for the passage of hydrophilic substances across the outer membrane. In this paper it is demonstrated that protein F is probably only a single polypeptide chain which is modified by 2-mercaptoethanol and by lengthy boiling in SDS. Furthermore, we define two new major polypeptide bands and four new heat-modifiable proteins, and we demonstrate the optimum conditions for examination of the *P. aeruginosa* outer membrane proteins by SDS-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Bacterial strains. Two prototrophic strains of *P. aeruginosa* PAO1 were principally used in this study; one, strain H101, was described previously and was obtained from A. J. Clark (10), and one, strain H103, was obtained from A. Kropinski. Some slight differences in minor outer membrane protein bands were observed, but otherwise the strains were similar. *P. aeruginosa* PAO37 strain AK2 (*argC4 chl-2* FP⁻) and its phage E79-resistant derivative strain AK43 were received from A. Kropinski (14) and were used in some experiments.

Medium and culture conditions. Proteose peptone no. 2 (1% [wt/vol]) (Difco 0121-01)-0.5% NaCl was used as a rich medium, and BM2 minimal medium (7), supplemented with 0.5 mM MgSO₄, 10 μM FeSO₄, and either 0.4% glucose or 20 mM potassium succinate as a carbon source, was used as a defined medium. Otherwise culture conditions were as described previously (10).

Outer membrane separation. Two methods were used for outer membrane separation. One, as previously described (10), yielded two outer membrane fractions with identical protein compositions, OM1 and OM2. The other method was a rapid method which separated membranes into two bands, one of which was purified outer membrane (OM1 + OM2). The procedure followed was similar to the previously published procedure (10), except that only one sucrose density gradient centrifugation was performed. Broken cells in 20% (wt/vol) sucrose-30 mM Tris-hydrochloride (pH 8.0) were layered onto a two-step gradient with 4 ml of 70% (wt/vol) sucrose in the bottom layer and 4 ml of 60% (wt/vol) sucrose in the top layer. This was centrifuged at 183,000 × *g* for at least 2 h (longer centrifugation improved the yields) in a Beckman SW41 Ti rotor. The lower outer membrane band was collected and washed free of sucrose, and its identity was determined as previously described (10).

SDS-polyacrylamide gel electrophoresis. Two basic methods were used to analyze *P. aeruginosa* outer membrane proteins. The method of Neville (22) was adapted to slab gel electrophoresis by using his buffer systems in the gel and running buffers (including the pH 9.18 lower gel and reservoir buffer). The acrylamide concentrations in the lower running gel were 14% (wt/vol) acrylamide and 0.12% (wt/vol) *N,N'*-methylenebisacrylamide, and the gel was polymerized by the addition (per 15 ml of gel mix) of 21 μl of TEMED (*N,N,N',N'*-tetramethylethylenediamine) and 0.3 ml of 1% (wt/vol) ammonium persulfate. In addition, the lower running gel contained 0.07 M NaCl to improve separation of lower-molecular-weight proteins (5). The upper or stacking gel was as described by Neville with the exception that the gel was polymerized by the addition (per 5 ml of gel mix) of 10 μl of TEMED and 0.12 ml of 1% (wt/vol) ammonium persulfate. Solubilization was accomplished by the dilution of the sample in an equal volume of solubilization reduction mix containing 4% (wt/vol) SDS, 10% (vol/

vol) 2-mercaptoethanol, twofold-concentrated upper gel buffer, and 20% (vol/vol) glycerol, followed by heating at various temperatures using a Temp-Block module heater (Lab Line Instruments Inc., Melrose Park, Ill.) filled with glycerol.

The other gel system used standardly to analyze *P. aeruginosa* proteins in this study was as described by Lugtenberg et al. (15) with two exceptions. Specially pure SDS from BDH (BDH Chemicals, Toronto, Canada; no. B30176) was utilized instead of low-grade SDS. Three different running gel acrylamide concentrations were used, with acrylamide-bisacrylamide ratios (all percent, wt/vol) of 14:0.255, 11:0.20, and 9:0.16. The 14:0.255 gels were supplemented in the running gel with 0.07 M NaCl. The gel system of de Jong et al. (4) (acrylamide-bisacrylamide, 15:0.4) was used in certain experiments. In addition, the following systems were tried without any improvement in the resolution obtained by the above methods. These were (i) the Lugtenberg et al. (15) system, substituting Biorad (Mississauga, Ontario) SDS or sodium deoxycholate for BDH SDS or with the addition of 0.1% (wt/vol) Triton X-100 to the gel and solubilization solutions, (ii) the slab gel system of Mizuno and Kageyama (18), and (iii) the SDS-urea gel system of Uemura and Mizushima (31).

The effect of various cations was tested by the addition of the cation directly to the solubilization mix before heating. The effect of lipopolysaccharide (LPS) was demonstrated by addition of LPS subsequent to heating of the sample in solubilization mix.

Purification of the porin protein. The technique used to purify the porin protein was a modification of the technique of Inouye et al. (13) for isolating free-form lipoprotein of *E. coli* B. Outer membranes were isolated from *P. aeruginosa* AK43 and solubilized in 5 ml of 2% SDS-25 mM Tris-hydrochloride buffer (pH 8.0)-10 mM EDTA for each gram (wet weight) of starting cells, by heating to 90°C for 20 min. The solution was cooled to room temperature, 13% (vol/vol) *n*-butanol was added, and the solution was placed in an ice bath. A solution of 4 N sodium acetate (pH 5.5) was added to 0.1 N, and, after the solution stood for 30 min in the ice bath, the resultant precipitate was removed by centrifugation (27,000 × *g*, 10 min). To the supernatant, 5% (vol/vol) acetone was added; after the solution stood for 1 h at 30°C, the precipitate was removed by centrifugation (27,000 × *g*, 10 min). This precipitate contained mainly lipoprotein (protein I) and some protein F. It was used by Mizuno and Kageyama to purify the lipoprotein (18). The supernatant was made 20 mM for MgCl₂ and then stood overnight at 4°C, after which the precipitate was removed by centrifugation (27,000 × *g*, 10 min). This supernatant was rich in protein F. In one experiment the acetone and Mg-acetone precipitates were pooled, resuspended in 1% SDS-10 mM Tris-hydrochloride (pH 7.4)-5 mM EDTA-50 mM NaCl (column buffer), and run on a column (9 by 45 cm) of Sephadex G150 eluted with column buffer. The peak that ran immediately after the void volume was substantially purified protein F (see Fig. 1, gel E).

Assays and chemicals. LPS was assayed by determining 2-keto-3-deoxyoctonate by the method of Osborn et al. (23), assuming the 2-keto-3-deoxyocto-

nate was 2.5% (wt/wt) of the LPS. Protein was determined by the method of Schacterle and Pollack (26) or, in the presence of SDS, by the method of Sander-mann and Strominger (25). Acrylamide and *N,N'*-methylenebisacrylamide were from Eastman Chemicals (Rochester, N.Y.); 2-mercaptoethanol and all other electrophoresis requirements came from Bio-Rad Laboratories (Mississauga, Ontario). All proteins used as standards, including bovine serum albumin (67,000 daltons), ovalbumin (45,000), carbonic anhydrase (30,000), α -chymotrypsinogen A (25,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000), were purchased from the Sigma Chemical Co. (St. Louis, Mo.). Sephadex G150 and G200 were purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

RESULTS

Glucose-induced outer membrane proteins. When *P. aeruginosa* PAO1 was grown on minimal medium in the presence of glucose, three major outer membrane proteins, D1, D2, and E, of very similar molecular weights were induced (Fig. 1, gel A). Proteins D1 and D2 were only separated on gels where the acrylamide concentration was 14% or greater. Protein D1 was not observed in the outer membranes of cells grown on proteose peptone no. 2 (Fig. 1, gels B to D) or tryptone yeast medium (10), whereas D2 and E were present in low amounts (Fig. 1, gels B to D). In contrast, the outer membrane of cells grown in minimal medium in the presence of succinate was only partially induced for these proteins (results not shown). Preliminary experiments demonstrated that the synthesis of D1 was suppressed by the presence of 0.1% proteose peptone no. 2 or 0.2% Casamino Acids, even in the presence of BM2 minimal medium and 0.4% glucose.

Stinson and co-workers (30) purified a periplasmic glucose-binding protein from *P. aeruginosa* and demonstrated a molecular weight of around 44,000 on an SDS-polyacrylamide gel. This protein was more strongly induced in the presence of glucose than after growth in succinate. To determine whether this protein was one of the above proteins, we followed the purification procedure of Stinson et al. (30), omitting only the last two steps. At this stage only one major band of apparent molecular weight higher than our glucose-induced outer membrane protein (i.e., approximately 53,000 on our gel system) was present in the solution. Although we did not test this protein for glucose-binding activity, we tentatively conclude that the glucose-binding protein is not identical to any of proteins D1, D2, or E.

Heat modifiability of SDS-solubilized outer membrane porin (protein F). Mizuno and Kageyama (18) observed that boiling of

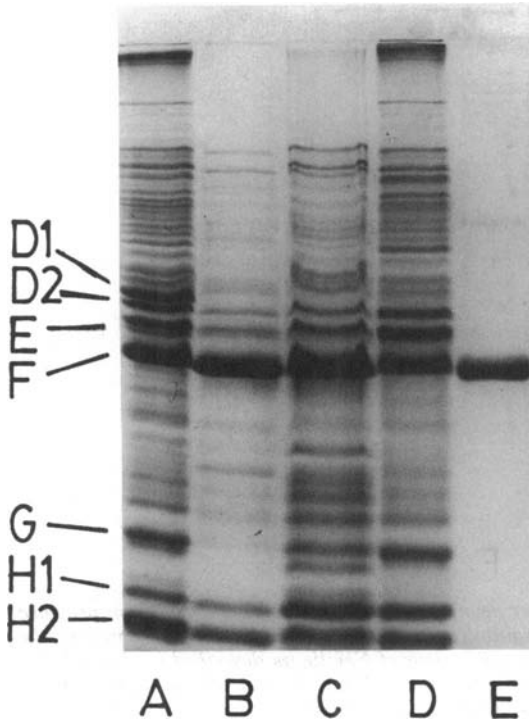


FIG. 1. Effect of growth conditions of *P. aeruginosa* outer membrane proteins solubilized in 2% SDS-5% 2-mercaptoethanol at 88°C for 10 min and run using the SDS-polyacrylamide 15% gel system of de Jong et al. (4). The gel was run for 166% of the time it took for the tracking dye to reach the end, to assist visualization of proteins D1 and D2. (A) Strain H103 grown in BM2 minimal medium with glucose as a carbon source; (B) strain AK43 grown in proteose peptone no. 2 medium; (C) strain AK2 grown in proteose peptone no. 2 medium (the band running close to the position of protein G is not protein G because [i] it has a slightly higher molecular weight and [ii] it is not heat modifiable); (D) strain H103 grown in proteose peptone no. 2 medium; (E) purified porin protein F.

outer membrane in SDS-2-mercaptoethanol for long periods of time caused an alteration in the mobility of the porin protein in outer membrane preparations from a faster (protein F) to a slower running form (F*). A similar shift from the F to the F* (37K) form was observed on phenol (10) or CCl₃COOH treatment (10, 18). We therefore probed this effect further using both outer membrane preparations and substantially purified porin preparations, and several different polyacrylamide gel systems. Parallel results were achieved by using both outer membrane preparations OM1 and OM2 or purified porin (Fig. 1, gel E); only results with whole outer membranes are illustrated in Fig. 2 and 3. Solubilization of outer membranes or purified porin at 88°C or

less for 10 min left all of the porin in the F form (Fig. 2, gels A to F; Fig. 3, gels A to F). Treatment in SDS-2-mercaptoethanol for 5 to 20 min at 100°C resulted in an increasing amount of the F* form, although the F form was always present (Fig. 2, gel G; Fig. 3, gel G). Treatment for 50 min to 1 h at 100°C usually resulted in most of the protein in the F* form (Fig. 2, gel H). Although, in some experiments, the porin was 90% converted to the F* form after 20 min of heating at 100°C in SDS-2-mercaptoethanol, this was not consistently observed, and in some experiments considerably longer periods of heating at 100°C were required for an equivalent conversion to the F* form, even in the case of the purified porin preparations. However, phenol or CCl₃COOH pretreatment consistently resulted in all of the porin, whether purified or outer membrane-associated porin, running as the F* form even after solubilization at only 60°C in SDS-2-mercaptoethanol.

An attempt was made to probe the F to F* conversion by chromatography on Sephadex G200 in the presence of 1% SDS-5 mM EDTA-10 mM Tris-hydrochloride (pH 7.4)-50 mM NaCl. As expected, only a very slight shift in the major protein peak was observed. However, this experiment allowed us to test a hypothesis advanced in a previous paper (10), that the protein F is bound to lipopolysaccharide (LPS) and pretreatment with CCl₃COOH or phenol (or heating in SDS) releases the LPS, allowing conversion to the F* form. Prior to column chromatography, the partially purified porin, which was 100% in the F form, contained 5.9 mg of LPS per mg of porin. After one cycle of column chromatography, most of the LPS was separated from the porin (still in the F form), which now had 0.32 to 1.12 mg of LPS per mg of porin. When the porin was heated for 1 h at 100°C in SDS prior to column chromatography, we still found 1.24 mg of LPS per mg of protein associated with the main protein peak. A sample which had undergone two cycles of column chromatography still contained porin in the F form, but had less than 0.07 mg of LPS per mg of porin associated with it. LPS-depleted protein F could be normally converted to the F* form by heating or phenol treatment as described above.

Heat modifiability of other outer membrane proteins. Four other outer membrane proteins were observed to be heat modifiable in the presence of SDS. Although these proteins were not purified in this study (but were partially purified previously [9]), by taking advantage of certain properties of the proteins and of certain mutants it was possible to unambiguously identify them at all solubilization temper-

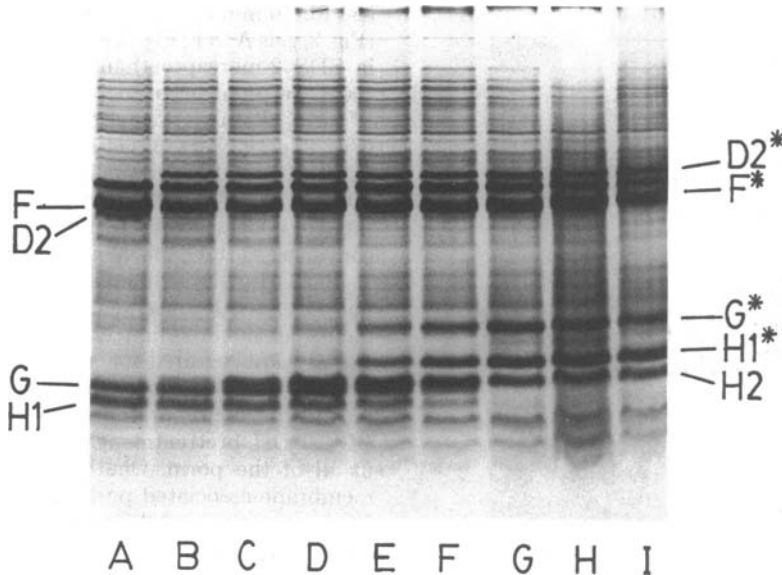


FIG. 2. Heat modifiability of *P. aeruginosa* H103 outer membrane proteins. Cells were grown on proteose peptone no. 2, and the outer membrane was purified, solubilized in 2% SDS-5% 2-mercaptoethanol at the indicated temperatures, and run on the 14% acrylamide gel system of Neville as described in the text. (A) Solubilized at 37°C for 10 min; (B) 48°C, 10 min; (C) 60°C, 10 min; (D) 73°C, 10 min; (E) 82°C, 10 min; (F) 88°C, 10 min; (G) 100°C, 10 min; (H) 100°C, 20 min; (I) 100°C, 50 min (in this sample F was 100% converted to F* as verified on a 9% SDS-polyacrylamide gel). The heat-modified forms of the proteins are distinguished from the unmodified forms by the addition of an asterisk (e.g., F*).

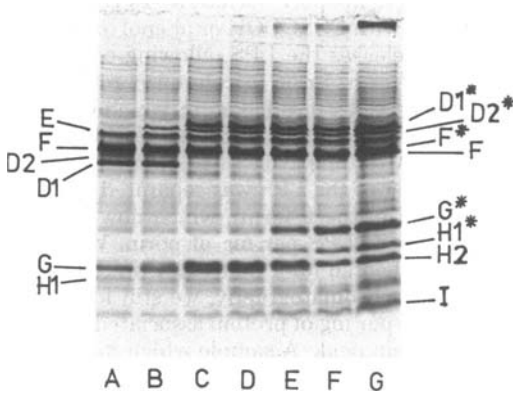


FIG. 3. Heat modifiability of *P. aeruginosa* H103 outer membrane protein. After growth of cells on BM2 minimal medium with glucose as a carbon source, outer membranes were purified, solubilized in 2% SDS-5% 2-mercaptoethanol at the indicated temperatures, and run on the 14% acrylamide gel system of Lugtenberg et al. (15) as described in the text. (A) Solubilized at 37°C for 10 min; (B) 50°C, 10 min; (C) 65°C, 10 min; (D) 75°C, 10 min; (E) 88°C, 10 min; (F) 100°C, 5 min; (G) 100°C, 30 min. The heat-modified forms are distinguished from the unmodified forms by the addition of an asterisk (e.g., D1*).

TABLE 2. Apparent molecular weights on 14% SDS-polyacrylamide gel electrophoresis of heat-modifiable proteins after solubilization at varying temperatures in the presence of 2% SDS-5% 2-mercaptoethanol

Protein	Apparent mol wt (10 ³)		Transition temp (°C)
	Low temp	High temp	
D1	35.5	46	50-56
D2	35.5	45.5	50-56
F (= porin)	39	41	100 (50 min)
G	19	25	75-82
H1	18.2	21	65-73
H2	20.5	20.5	—
I (= lipoprotein)	9.0-14	9.0-12	—

characterized by their presence in the outer membranes of cells grown in minimal medium in the presence of glucose and by their virtual absence in the outer membranes of cells grown in proteose peptone no. 2 medium (Fig. 1, gels A and D). A shift of 10,000 in the apparent molecular weight of D1 and D2 was observed when the temperature of solubilization was shifted from 50°C to 60°C (bands D1* and D2*, see Fig. 3, gels B and C).

It was observed that protein G was fortuitously absent in *P. aeruginosa* PAO37 (see Fig.

atures. The results below are summarized in Table 2.

The glucose-induced proteins D1 and D2 were

1, gel C). This enabled monitoring of the change in mobility of protein G with temperature of solubilization in SDS from a diffuse band of apparent molecular weight of 19,000 at 75°C to a sharp band of 25,000 at 88°C (band G*, see Fig. 2 and 3).

During this study it was observed that protein H, previously observed as a single band (see Table 1), ran as two closely spaced bands on SDS-polyacrylamide gels with an acrylamide monomer concentration greater than 14% (see, e.g., Fig. 2, gel F). Furthermore, these polypeptides differ in that only one is heat modifiable: protein H2, of lower apparent molecular weight, is stable to heating in SDS. Protein H1 was identified by its hyperproduction in two strains resistant to polymyxin B (T. Nicas, unpublished data). Using either of these mutants (results not shown) or the wild-type strain PAO1, we demonstrated that heating in SDS at temperatures greater than 73°C caused an alteration in the mobility of the protein (to band H1*, see Fig. 2, gel E). No other outer membrane proteins were demonstrated to be heat modifiable.

Effects of LPS and cations on the electrophoretic mobility of heat-modifiable proteins. Schweizer et al. (28) demonstrated that LPS reversed the heat modification of protein II/II* in *E. coli* K-12, although the LPS did not remain associated with the protein during electrophoresis. It was shown that addition of LPS to heat-modified proteins D1*, G*, and H1* resulted in these proteins running at lower molecular weights. It required a weight ratio of LPS to protein of 1:1 to cause such changes, and this caused a marked alteration in the electrophoretic protein patterns, including the shift of all proteins larger than 50,000 daltons and protein E to another running position. Therefore we were able to conclude definitely that proteins D1*, G*, and H1* were shifted back to the heat-unmodified positions. However, it was possible to conclude from experiments using both whole outer membranes and purified porin that LPS had no effect on protein F* mobility, and that furthermore there was no apparent effect on the electrophoretic mobility of proteins H2 or I, even when twice as much LPS was added subsequent to solubilization.

McMichael and Ou (17) demonstrated that Tris buffer (pH 6.8), MgCl₂, and NaCl could inhibit the conversion of protein II to its heat-modified form in *E. coli* K-12. No such effects were observed for proteins D1, F, or H1 of *P. aeruginosa* outer membranes, whereas 1 to 100 mM MgCl₂ partially inhibited conversion of G to G* at 88°C. NaCl (0.3 M) had no effect on any of the proteins tested. However, when ZnSO₄ or (to a lesser extent) MgCl₂ was added

during solubilization, the transition of protein D1 to its heat-modified form was promoted at 45°C. During this study, we observed that the inclusion of 0.1 M ZnSO₄ or 0.1 M MgCl₂ greatly increased the amount of lipoprotein (protein I) in SDS-polyacrylamide slab gels. Normally this protein was not well stained, due to either failure to enter the gel or extraction during fixation and staining of the gel.

The effect of 2-mercaptoethanol on the apparent molecular weight of the porin. 2-Mercaptoethanol, which reduces cystine disulfide bonds, is routinely added during the solubilization of samples in SDS prior to polyacrylamide gel electrophoresis. To determine whether disulfide bonds were present in outer membrane proteins, 2-mercaptoethanol was omitted from the sample solubilization mix prior to electrophoresis.

Pretreatment with CCl₃COOH caused a shift in the apparent molecular weight of the porin protein of the outer membrane, both in the presence (10, 18; Table 3) and absence (Table 3) of 2-mercaptoethanol. This was confirmed using two purified protein F preparations (Table 3). It was observed that the omission of 2-mercaptoethanol caused an increase in the mobility (i.e., a lower apparent molecular weight) of the porin protein for both CCl₃COOH-pretreated and non-treated outer membranes and purified porin preparations (Table 3). As summarized in Table 3, footnote a, CCl₃COOH pretreatment caused an apparent molecular weight shift of 6,000 upwards, whereas 2-mercaptoethanol caused an apparent increase of 3,000. These data suggested that these treatments were not affecting the same portions of the porin protein, since the effects were additive. No other major protein

TABLE 3. Apparent molecular weight of the *P. aeruginosa* PAO1 porin on a 9% SDS-polyacrylamide gel after various solubilization procedures^a

Solubilization procedure			Samples	Apparent mol wt
SDS	2-Mercaptoethanol	CCl ₃ COOH pretreatment		
+	+	-	A, B, C	36,000
+	+	+	D, E, F	42,000
+	-	-	H, I, J	33,000
+	-	+	K, L	39,000

^a Whole outer membranes of strain H103 grown in BM2 glucose were run as samples A, D, H, and K; two different purified preparations were run as samples B, E, I, and L and samples C, F, and J, respectively. The effective separation range of the 9% gel was from 28,000 daltons to approximately 200,000 daltons. Thus all major proteins of less than 28,000 daltons ran as a single band coincident with the dye front.

bands were affected by the omission of 2-mercaptoethanol.

When the concentration of the 2-mercaptoethanol during solubilization was varied from 0 to 5% (vol/vol), porin protein F underwent two clearly defined alterations in mobility on subsequent SDS-polyacrylamide gel electrophoresis (Table 4). A concentration of between 0.2 and 0.5% (vol/vol) 2-mercaptoethanol was required during solubilization for all of the protein to run as a single band at an apparent molecular weight of 36,000. Similar results were observed for the heat- or CCl_3COOH -modified F^* form of the porin protein. Increasing 2-mercaptoethanol concentrations from 0 to 0.5% (vol/vol) caused symmetrical shifts in the apparent molecular weights of both the F and F^* forms (Table 4), again suggesting that the F to F^* transition was affecting a different portion of the porin protein. The shift in the apparent molecular weight of the protein F caused by 0.5% 2-mercaptoethanol was not reversed by the addition of 0.1 M MgCl_2 , suggesting that the 2-mercaptoethanol was not merely acting as a chelator (17).

DISCUSSION

Although the advent of two-dimensional polyacrylamide gel electrophoresis has enabled efficient analysis of complex mixtures of proteins, it suffers from one major disadvantage, especially with respect to outer membrane proteins: a single gene product can produce a number of dis-

TABLE 4. Apparent molecular weight(s) of protein F and its heat-modified form, F^* , after solubilization in SDS at various 2-mercaptoethanol concentrations

2-Mercaptoethanol concn ^a (%)	Form of protein F	Apparent mol wt ($\times 10^3$)					
		33	34.5	36	39	40.5	42
0	F	+	-	-	-	-	-
0.001	F	+	+	-	-	-	-
0.005	F	+	+	-	-	-	-
0.01	F	+	+	-	-	-	-
0.025	F	-	+	+	-	-	-
0.1	F	-	+	+	-	-	-
0.5	F	-	-	+	-	-	-
0	F^*	-	-	-	+	-	-
0.001	F^*	-	-	-	+	+	-
0.005	F^*	-	-	-	+	+	-
0.01	F^*	-	-	-	+	+	-
0.025	F^*	-	-	-	-	+	+
0.1	F^*	-	-	-	-	+	+
0.5	F^*	-	-	-	-	-	+

^a Concentration is given as percent (vol/vol) final concentration during the heating at 88°C or higher in 2% SDS prior to polyacrylamide gel electrophoresis on a 9% Lugdenberg gel (see the text). A number of intermediate and higher concentrations of 2-mercaptoethanol were tested; only representative results are presented.

tinct polypeptide spots with different isoelectric points and apparent molecular weights (11). One-dimensional SDS-polyacrylamide gel electrophoresis has been a powerful tool for the study of *E. coli* outer membrane proteins, particularly since most outer membranes have a small number of proteins present in large amounts. The major setbacks of one-dimensional SDS-polyacrylamide gel electrophoresis (which are, of course, also problems in two-dimensional gel electrophoresis) have been, first, resolution, and, second, the presence of heat-modifiable proteins (6). Resolution is a problem since two or more distinct polypeptide chains may appear as a single protein band, whereas heat-modifiable proteins can appear as two distinct polypeptide bands depending on the temperature of solubilization (see, e.g., Fig. 3, gel G). In this paper we have attempted to define the number and nature of major outer membrane polypeptide bands of *P. aeruginosa* while at the same time probing the unusual properties of these proteins.

It was found that two of the major outer membrane protein bands previously defined by four groups (2, 10, 16, 18) were separated into two narrowly spaced bands when run on SDS-polyacrylamide gels with acrylamide monomer concentrations greater than 14%. Proteins D1 and D2 are heat-modifiable proteins of similar apparent molecular weight both in the heat-modified and unmodified forms. They were strongly induced in minimal medium with glucose as a carbon source. Protein D1 was not observed at all in cells grown in rich medium, whereas protein D2 was at a very low level. Another major protein E, although not heat modifiable, was somewhat induced in glucose minimal medium. Since other proteins associated with glucose transport and metabolism are induced in glucose minimal medium (30), it is possible that these proteins are involved in passage of glucose across the outer membrane in the same way that the lambda receptor is associated with maltose uptake in *E. coli* K-12 (6).

Proteins H2 and G were also heat modifiable. We were able to identify them at all solubilization temperatures by using mutants. As yet, we are unable to determine the function of these proteins. It has been demonstrated that strain PAO37, which lacks protein G, is fully sensitive to 41 bacteriophages and 17 pyocins capable of lysing *P. aeruginosa* PAO1.

Mizuno and Kageyama (18) demonstrated that protein F was heat modifiable by using whole outer membranes. We have confirmed that this is an intrinsic property of the protein using purified protein F. The shift in mobility of

protein F on SDS-polyacrylamide gels is also caused by pretreatment with CCl_3COOH or phenol and subsequent removal of these agents before SDS solubilization (10, 18; Table 3). We previously postulated (10) that the F form of protein F was LPS associated and that removal of LPS by the above pretreatments results in the protein running at a higher apparent molecular weight (i.e., F^*). However, we have now demonstrated that purified porin protein containing less than 7% (wt/wt) associated LPS was still present in the F form and could be normally converted to the F^* form. Thus LPS association cannot be the difference between the F and F^* forms.

The migration of proteins in SDS-polyacrylamide gel electrophoresis is directly related to the amount of SDS bound. Other factors such as the charge (4) and the conformation of the SDS-protein complexes (and hence the frictional drag [4]) can also influence the mobility. It has been demonstrated that the heat-modifiable outer membrane proteins of *E. coli* K-12 contain β -structured sequences which are stable in SDS solution, but which are destroyed upon heating (20, 21). Our results with proteins D1, D2, G, and H1 are consistent with results for protein 0-10 (II^*) of *E. coli*, in that heating in SDS at temperatures around 70 to 100°C caused a decrease in the mobility of these proteins (i.e., an increase in the apparent molecular weight). Furthermore, addition of LPS to heat-modified D1^* , D2^* , H1^* , and G^* reverses the heat modification, much as it does with protein 0-10 (II^*) of *E. coli* K-12. Although there are minor differences in the response of these proteins to cations, we tentatively conclude that they fall into a single class of heat-modifiable proteins. The small change in the mobility of these proteins suggests that only a small region of the protein molecule is responsible for the effects described above and that the major part of the polypeptide chain interacts normally with SDS at all temperatures. This is probably also true for protein F, although, unlike the above proteins, long periods of boiling in SDS are required to effect conversion of the protein to the F^* form. In addition, the protein is quite stable to a number of treatments which affect the other heat-modifiable proteins. Further study is required before the bases for such differences are understood, but it seems unlikely that the presence of β -structured polypeptide is solely responsible for the anomalous behaviour of outer membrane proteins on heating in SDS. It should, however, be mentioned that heat modifiability is a cooperative effect, since bands of intermediate mobility are never observed.

The heat modifiability of *P. aeruginosa* outer membrane proteins was observed in both the presence and absence of 2-mercaptoethanol. This suggested that the change in mobility caused by the addition of 2-mercaptoethanol was affecting a part of the molecule different from the heat-modifiable portion, especially since the two modifications were of different magnitudes. The simplest explanation for the effect of 2-mercaptoethanol is that it is affecting one or possibly two cystine disulfide bonds. Since the mobility of protein F (or its F^* form) is decreased by 2-mercaptoethanol treatment, the alteration is probably due to the breaking of intrachain (rather than interchain) disulfide bridges. This would cause an alteration in the conformation of the protein and thus increase the binding of SDS (or the frictional drag) of the molecule. If this explanation proves true, this is the first example of a membrane protein with an intrachain disulfide bond of which we are aware. None of the major proteins of the *E. coli* K-12 outer membrane forms disulfide bridges, despite the presence in one of them (protein II^* ; reference 8) of two half-cystines. We are currently probing the influence of 2-mercaptoethanol on the function of this protein as a porin.

As described above, the apparent number and mobility of the major outer membrane protein bands of *P. aeruginosa* are influenced by the concentration of acrylamide monomer (Table 1), the temperature and duration of solubilization in SDS (Fig. 2 and 3), the concentration of 2-mercaptoethanol during solubilization (Tables 3 and 4), and the type of pretreatment prior to solubilization (e.g., Table 3). Thus, using the 8% SDS-urea polyacrylamide gel system of Uemura and Mizushima (31), proteins G, H1, and H2 run as a single protein band. In contrast, we have observed conditions under which protein F will appear as four separate bands, and there are a total of six distinct dodecyl sulfate-protein F complexes which can appear according to the solubilization conditions (Table 4). For future studies of *P. aeruginosa* outer membrane proteins, we recommend the solubilization of protein in 2% SDS-5% 2-mercaptoethanol at 85 to 95°C for 10 min, followed by running on the 14% acrylamide-SDS-polyacrylamide gel system (based on the technique of Lugtenberg et al. [15]) as described in Materials and Methods. Under these conditions, the major proteins band sharply and in what appear to be unique positions (since we can see no significant protein bands in these positions before heat modification). Only protein F is not heat modified at this solubilization temperature. As described above, the lipoprotein (protein I) will appear strongly

in the gel if 0.1 M MgCl₂ is added during solubilization of the sample.

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