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Outer Membrane Porin Proteins F, P, and D1 of *Pseudomonas aeruginosa* and PhoE of *Escherichia coli*: Chemical Cross-Linking to Reveal Native Oligomers

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Native oligomers of three *Pseudomonas aeruginosa* outer membrane porin proteins and one *Escherichia coli* porin were demonstrated by using a chemical cross-linking technique. *P. aeruginosa* protein F, the major constitutive outer membrane porin, was cross-linked to dimers in outer membrane and whole-cell cross-linking experiments. Purified preparations of *P. aeruginosa* proteins F, D1 (glucose induced), and P (phosphate starvation induced) and *E. coli* protein PhoE (Ic) were also cross-linked to reveal dimers and trimers upon two-dimensional sodium dodecyl sulfate-polyacrylamide electrophoretic analysis. Cross-linking of protein F was abolished by pretreatment of the protein with sodium dodecyl sulfate, indicating that the cross-linked products were due to native associations in the outer membrane.

Major outer membrane proteins called porins of *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* have been shown to form passive diffusion pores in reconstituted outer membrane vesicles (5, 12, 13, 26) and when tested in the black lipid bilayer system (3, 14). In vivo evidence for the function of porins as nonspecific hydrophilic channels has also been afforded by mutants lacking the porins of *E. coli* and *S. typhimurium* (2, 27) or lacking protein F of *P. aeruginosa* (25), in which permeability of the outer membrane to β-lactam compounds was found to be reduced compared with wild-type strains.

The porins examined in our study are of major interest because of several similarities and dissimilarities with *E. coli* porins previously examined. Protein F of *P. aeruginosa* forms larger water-filled pores in vitro than do the major porins of *E. coli* (3, 13), yet the permeability of *P. aeruginosa* to hydrophilic antibiotics in vivo is much lower (1, 25). This phenomenon has been ascribed to having <1% of protein F forming functional channels (3).

Protein P and *E. coli* PhoE are both induced under low phosphate conditions, are apparently coregulated with alkaline phosphatase, form anion-selective channels in model membranes, and form sodium dodecyl sulfate (SDS)-resistant oligomers on SDS-polyacrylamide gels (17, 28, 36). These proteins differ, however, in their apparent molecular weights, apparent pore size in black lipid bilayers, with protein P channels being substantially (three-fold) smaller than those of PhoE, and degree of selectivity toward anions, with protein P being almost anion specific (14). Protein D1 has been compared to the LamB protein of *E. coli* due to the proposed involvement of both in periplasmic binding protein-mediated carbohydrate uptake systems and due to their similar mode of induction (6, 11).

On SDS-polyacrylamide gel electrophoresis, the major outer membrane porin proteins of *E. coli* and *S. typhimurium* are present as oligomers, specifically trimers, when solubilized without heating (16, 23, 42). The major outer membrane porin protein of *P. aeruginosa*, protein F, does not show oligomers upon SDS-polyacrylamide gel electrophoresis, even when solubilized without heating, possibly due to its sensitivity to SDS, which is known to abolish pore-forming activity of protein F in vitro (3).

Native oligomers of *E. coli* outer membrane proteins OmpF, OmpC, OmpA, and LamB were also shown to exist by chemical cross-linking of both outer membrane and purified protein preparations (29–34). These proposed trimer structures have since been confirmed by sedimentation equilibrium experiments (23). Therefore, by implication, cross-linking is a valuable method for the study of native multimeric associations in outer membranes. In the case of *P. aeruginosa* protein F, cross-linking was the only method available to our group because sensitivity of the protein to SDS and its copurification with lipopolysaccharide (LPS) would have made sedimentation equilibrium analysis difficult to interpret.

Chemical cross-linking has also been widely used in a variety of systems to demonstrate oligomeric associations of proteins (7, 35, 39). For rapid and efficient cross-linking with reagents of the type used in this study, the necessary functional groups in the protein (primarily amines, although the imidazole group of histidine and cysteine sulfhydrals can react [35]) must be both accessible and properly oriented for cross-linking to occur. Once multimers are identified, the composition or stoichiometry of a cross-linked product is usually evaluated by (i) the molecular weights estimated on the basis of relative electrophoretic mobility, (ii) the alignment of cleaved components on two-dimensional gels, and (iii) the relative intensities of the bands (35). Some of the potential problems include the fact that excess cross-linking reagent can lead to monofunctional reactions and adverse effects (35), resulting in generation of blurred bands (30), aggregates of oligomers even with purified polypeptides (7, 32), and bands which do not apparently correspond to a multimeric product (30, 32). Therefore, generally, conditions are selected such that minimal cross-linking is observed. Thus, although chemical cross-linking can provide an excellent demonstration of the existence of native oligomers, it cannot be used as sole proof of a given oligomeric association.

In this study, a cleavable cross-linker was used to try to establish whether *P. aeruginosa* porins and the *E. coli* PhoE protein were present as oligomers in their native forms. The cross-linker, dithio-*bis*-(succinimidyl propionate) (DSP), provides a bridge length of approximately 1.2 nm and therefore should only react with subunits in close proximity. The cleavability of DSP by disulfide reducing agents also makes it suitable for two-dimensional analysis of outer membrane proteins. Using this reagent, we have obtained evidence that these porins are arranged as oligomers in the outer membrane.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *P. aeruginosa* PAO1 strain H103 used for all cross-linking experiments was a prototroph (24). Cells were grown in BM2-minimal glucose medium (11) for all whole-cell and outer membrane experiments. In addition, cells were grown in BM2-minimal glucose medium for isolation of protein D1, in Proteose Peptone no. 2 (Difco Laboratories, Detroit, Mich.) medium for isolation of protein F, and in phosphate-deficient medium (14) for isolation of protein P. The *E. coli* strain used for isolation of protein PhoE was CGSC 6067, *proC24 ompF254 his-53 ompC171, nmpA104, metA104, metB65, lacY29, xyl-14, rpsL97, cycA1, cycB2, tsx-63,* $-\lambda^-$ (8), and was obtained from B. Bachmann of the Coli Genetic Stock Centre, Yale University, New Haven, Conn.

Chemicals. The cross-linker DSP was obtained from Pierce Chemical Co., Rockford, Ill. Disulfide reducing agents dithioerythritol and 2-mercaptoethanol were purchased from Bio-Rad Laboratories, Richmond, Calif.

Cell fractionation and protein purification. Outer membranes were prepared by the method of Hancock and Carey (10). Proteins F (3), D1 (11), and P (14) were purified as stated in previous publications. Protein PhoE was purified by using the same protocol as for protein P. Proteins P and PhoE were purified in our laboratory by K. Poole and R. Darveau, respectively, and were active in lipid bilayer reconstitutions of pore-forming activity (R. Benz, personal communication).

Cross-linking of outer membranes and purified proteins. Outer membrane and purified protein preparations were cross-linked in 15- to 20-μl volumes of 0.2 M triethanolamine buffer (pH 8.5), following the specifications of Reithmeier and Bragg (34). The cross-linker DSP was dissolved in dimethyl sulfoxide and added to the samples as a 1/100 dilution to give the optimum final concentration of DSP as indicated for each experiment in Results. After 2 min of reaction time, excess 1 M Tris-hydrochloride (pH 8.5) was added to stop the cross-linking reaction, and the preparations were diluted 1:1 into sample buffer containing 4% (wt/vol) SDS, 0.5 M Tris-hydrochloride (pH 6.8), and 20% (vol/vol) glycerol without reducing agent.

Gel electrophoresis. Samples were heated for 10 min at 88°C and then run on SDS-polyacrylamide slab gels (with various acrylamide concentrations as indicated for each experiment) as previously described (10, 22). The appropriate lanes were cut out and soaked for 15 min in sample buffer with reducing agent (either 10% [vol/vol] 2-mercaptoethanol or 10 mM dithioerythritol) to break the DSP molecules at their internal disulfide bond, placed atop another slab gel of the same percent acrylamide, and sealed in place with 0.8% (wt/vol) agarose. The second dimension was then run as described above at 130 V constant voltage. First-dimension gels were stained with Coomassie blue (Serva R250) and second-dimension gels were stained by the more sensitive (approximately fivefold) silver-staining method of Wray et al. (40). In this system, proteins not cross-linked ran along a diagonal; i.e., they had the same electrophoretic mobility (apparent molecular weight) in both the first and second dimensions, whereas cross-linked proteins appeared as spots below the diagonal on the second-dimension gel.

Whole-cell cross-linking. Whole-cell cross-linking experiments were performed as described by Palva and Randall (31) by concentrating logarithmic-phase cells 100-fold to give approximately 10^{10} cells per ml in a volume of 1 ml. After centrifugation, outer membrane permeability was not altered (1) and cells were motile, suggesting no appreciable disturbance of the cell surface. Cells were cross-linked by the addition of 10 to 100 μg of DSP per ml and reacted for 2 min, and then the reaction was terminated with excess 1 M Tris-hydrochloride (pH 8.5). Outer membranes were isolated as described previously (10) and analyzed by two-dimensional SDS-polyacrylamide gel electrophoresis.

RESULTS

Cross-linking of PhoE protein. Protein PhoE of *E. coli* has been recently shown to be a pore-

forming protein which is three- to fourfold selective for anions over cations (17; Benz and Hancock, manuscript in preparation). In the unheated form on SDS-polyacrylamide gels, it forms an oligomer with an aberrant electrophoretic behavior similar to that of other *E. coli* porins (23, 28). In addition, the structural gene has been isolated by Tommassen et al. and shown to demonstrate strong nucleic acid and primary amino acid sequence (64%) homology with the OmpF porin of *E. coli* (37). Therefore, it may be expected that this protein would be a trimer like the OmpF porin. This was confirmed by cross-linking and electrophoretic analysis in both one and two dimensions (Fig. 1).

When cross-linked purified PhoE preparations run on first-dimension SDS-polyacrylamide gels were treated with 2-mercaptoethanol to break the cross-link bridges and then run in the second dimension, evidence of multimers was observed in silver-stained gels (Fig. 1). Silver staining, which has been shown to be quantitative for given proteins (40), was preferred over [^{35}S]methionine labeling for visualization of multimers in the second dimension to avoid possible isotope effects on nearby DSP-reactive amino groups and potential effects of sulfate starvation on cells. However, the major spots seen by silver staining were confirmed to be proteinaceous, using both cross-linked [^{35}S]methionine-labeled outer membranes (for protein F) and Coomassie blue-stained gels (for all proteins). Streaks across gels at apparent molecular weights of 60,000 and 67,000 were enhanced by silver staining and were possibly due to impurities in one or more of the reagents used. These blemishes of two-dimensional polyacrylamide gel electrophoresis have also been reported by other authors (9).

Multimers appeared as spots below the diagonal line of uncross-linked proteins in Fig. 1 and were horizontally aligned to the protein from which they were derived. This confirmed that the higher-molecular-weight products seen in the Coomassie blue-stained one-dimensional gel were derived from the PhoE protein and were not artifacts. The purified 38,000-dalton monomer was freely cross-linked to form a 34,000-dalton band (probably due to an intrachain bridge), a dimer of 73,000 daltons, and a trimer of 100,000 daltons. In addition, a band of 140,000 estimated molecular weight was observed. Since large amounts of a similar product (called band C) were seen in cross-linking experiments with purified OmpF protein and explained by the authors as due to aberrant mobility in gels or as an aberrant conformation of an oligomer in SDS (32), we feel that this product may not represent a tetramer. Therefore, these studies demonstrated that our basic cross-linking techniques were

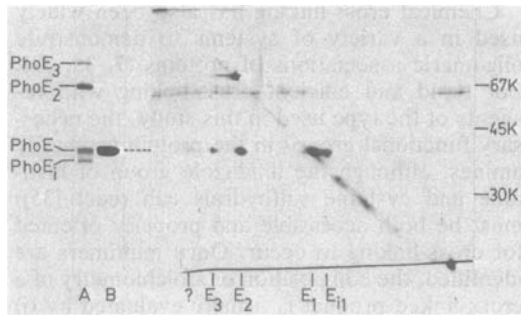

FIG. 1. One- and two-dimensional cross-linking analysis of purified *E. coli* protein PhoE. Purified protein PhoE was treated with 500 μg of DSP per mg of protein and run on an 11% polyacrylamide gel. The first-dimension gel was cut out and soaked in reduction mix containing 2-mercaptoethanol to break DSP cross-links, and then the lane was laid on top of another 11% acrylamide gel and run in the second dimension as described in the text. The first-dimension gel showed cross-linked products: lane A, PhoE + DSP; lane B, PhoE untreated control. Products observed in the first- or second-dimension gels or both were an internally cross-linked monomer (E_{i1}), dimers (E_2), trimers (E_3), and a small amount of higher-molecular-weight product(s) of unknown derivation. The running positions of the monomer of the PhoE protein in the first dimension and the monomer and spots derived from cross-linked oligomers in the second dimension are labeled on the left-hand side.

sound and suggested that PhoE, like other *E. coli* porins, was a trimer.

Cross-linking of protein P of *P. aeruginosa*. Protein P, like the PhoE protein, is induced by phosphate starvation. Unlike other *P. aeruginosa* porins (but like the PhoE protein), it forms stable oligomers on SDS-polyacrylamide gels after solubilization at low temperatures. It differs from the PhoE protein in that it has a higher apparent monomer molecular weight (Fig. 2), a smaller channel size, and a higher specificity for anions (i.e., it is 100-fold selective for anions over cations in black lipid bilayers [14]).

Four cross-linked products of the 48,000-dalton monomer of protein P (of estimated molecular weights 44,000, 80,000, 90,000, and 145,000) could be visualized (Fig. 2). The 44,000-dalton spot was presumably due to an intrachain cross-link which caused tighter folding of the protein and thus a lower apparent molecular weight. Similarly, the 80,000-dalton band was possibly a dimer of subunits with an intrachain cross-link. Based on molecular weight, the 90,000-dalton band was most likely a dimer of the 48,000-dalton protein P monomer. Another spot seen on both the one- and two-dimensional gels probably corresponded to a trimer of protein P, with an estimated molecular weight of 145,000. This

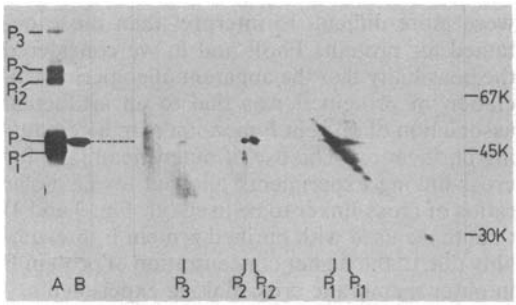

FIG. 2. One- and two-dimensional cross-linking analysis of purified *P. aeruginosa* protein P. Purified protein P was treated as in the legend to Fig. 1 except that a 9% polyacrylamide gel was used. Cross-linked products were observed in both the first- and second-dimension gels: lane A, first-dimension DSP cross-linked protein P; lane B, uncross-linked control. Products included a monomer of protein P_1 with an internal cross-link resulting in lower apparent molecular weight (P_{i1}), a dimer of protein P_1 (P2), a dimer with at least one internal cross-link (P_{i2}), and a trimer of protein P_1 (P3). The running positions of the monomer of protein P in the first dimension and the monomer and spots derived from cross-linked products in the two-dimensional gel are indicated on the left-hand side.

product was present in lower amounts in the gel than the trimer of the PhoE protein, possibly due to (i) insufficient cross-linker, (ii) a higher tendency to form intrachain cross-links (as evidenced by the large amount of the 80,000-dalton band for which no corresponding product was observed after cross-linking of the PhoE protein), or (iii) cross-linking to LPS which copurified with protein P and which has free amino groups in its O antigen (18). To rule out insufficient cross-linker as the reason, we added two-fold more cross-linker and still observed limited amounts of trimer on one-dimensional gels. At still higher concentrations of cross-linker most of protein P (and protein F [see below]) no longer entered the gel as monomers or oligomers, presumably due to the formation of SDS-insoluble complexes with cross-linker (the protein was probably not simply in the form of large cross-linked complexes since we did not observe protein staining in the stacking gel). This confirms the data of other workers who have shown that higher concentrations of cross-linker (up to 500-fold molar excess over protein concentration was used for protein P in the above case) can result in nonspecific cross-linking (7, 32, 35) and presumably saturation of amino groups on the protein.

Cross-linking of purified proteins F and D1. The purification procedure for protein F (involving solubilization in Triton X-100–EDTA) retained the protein's pore-forming activity in black lipid bilayers, and protein F was therefore presumed to be still in its native or active conformation. Cross-linking of purified protein F clearly revealed a dimer product of 70,000 daltons (Fig. 3). In addition, a smear of protein with an average molecular weight of 120,000 was observed. These oligomers were only seen in two-dimensional analyses, although broad areas of staining corresponding to dimer and trimer products were observed in silver-stained one-dimensional gels (i.e., before cleavage of cross-links). Cross-linker concentrations were varied over a 500-fold range (4 to 2,000 μg per mg of protein) for both purified protein F (not shown) and outer membranes (Fig. 4, 150-fold concentration range demonstrated), and time of treatment with cross-linker varied from 30 s to 30 min. At no cross-linker concentration were sharp oligomer bands seen in first-dimension gels, although the protein F monomer band disappeared at higher cross-linker concentrations (200 to 1,000 μg of DSP per mg of protein depending on the treatment times). At the same time, when the cross-linked products were run in the first dimension and then cleaved and run in the second dimension, broad spots with average molecular weights of 70,000 and 120,000 appeared as discussed above. The experiment demonstrated in Fig. 3 was performed at a concentration at which 60% of the protein F disappeared from the monomer position. We estimated that approximately 40% appeared at a molecular weight equivalent to dimer (F_2) and 20% appeared as higher oligomers including apparent trimers (F_3). The higher oligomers

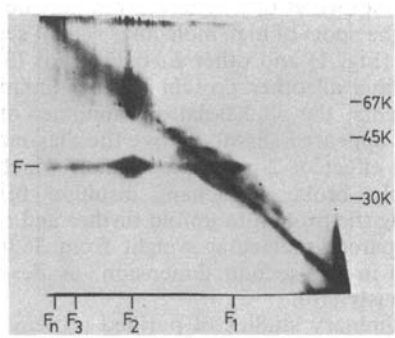

FIG. 3. Two-dimensional cross-linking analysis of purified *P. aeruginosa* protein F. Purified protein F was treated as in the legend to Fig. 1. Cross-linked products included dimers (F_2) and trimers (F_3) of protein F as well as higher-molecular-weight cross-linked products of protein F (F_n). The two monomer spots above the diagonal were due to incomplete 2-mercaptoethanol modification of some of the protein F.

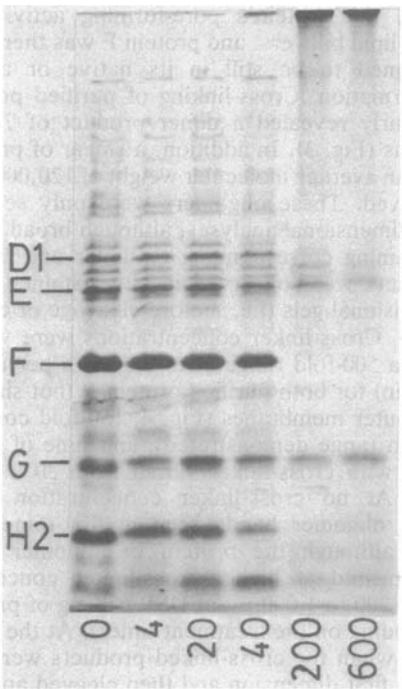

FIG. 4. One-dimensional SDS-polyacrylamide gel electrophoresis of outer membrane samples treated with various concentrations of DSP for 2 min. Cross-linked outer membranes were run on an 11% polyacrylamide gel as described in the text. Concentrations of DSP indicated beneath the individual lanes are expressed as micrograms per milligram of protein. Cross-linking was visualized in the first dimension as a decrease in staining intensity of protein bands and a concomitant increase in amount of high-molecular-weight material unable to enter the separating gel.

were not further characterized but may be related to the spots of high molecular weight seen for PhoE (Fig. 1) and other *E. coli* porins (32). In these and all other protein F cross-linking experiments, the 38,000-dalton monomer of protein F appeared slightly above the diagonal due to the effect of 2-mercaptoethanol, which presumably broke intrachain disulfide bridges, causing the protein to unfold further and raising its apparent molecular weight from 35,000 to 38,000 in the second dimension, as described previously (10).

Preliminary studies of purified protein D1, a glucose-inducible porin of *P. aeruginosa*, showed that it also existed as a multimer in its native form. Cross-linked products observed consisted of a dimer (95,000 daltons), possible trimer of 145,000 estimated molecular weight, and higher-molecular-weight complexes which did not enter an 11% gel (data not shown).

Cross-linking of protein F in outer membranes. Because the data obtained for purified protein F were more difficult to interpret than those obtained for proteins PhoE and P, we considered the possibility that the apparent oligomeric association of protein F was due to an artifactual association of protein F monomers induced during purification. The use of outer membrane for cross-linking experiments allowed lower molar ratios of cross-linker to be used (cf. Fig. 3 and 4) than those used with purified protein F, presumably due to the higher concentration of protein F in outer membrane cross-linking experiments.

At concentrations of cross-linker (40 μg of DSP per mg of protein) causing minimal alteration of outer membrane protein patterns (see Fig. 4), the only cross-linked product observed in two-dimensional analyses was a dimer of 70,000 daltons (Fig. 5B). With another cleavable cross-linker (dimethyl 3,3′-dithiobis-propionimidate) at higher concentrations, up to an estimated 20% of the monomer could be converted to dimer without the appearance of other cross-linked products in the analysis (data not shown). At a relatively high concentration of DSP (200 μg per mg of protein; note that the treatment time used here was less than that in Fig. 4), protein F was cross-linked to form visible dimers of 70,000 estimated molecular weight and a streak of higher-molecular-weight products (Fig. 5A), some due to multimers and some apparently due to associations with other proteins or LPS. To confirm that protein F could be cross-linked to LPS, a two-dimensional gel of cross-linked outer membranes was stained for LPS by the periodate-silver stain method of Tsai and Frasch (38), which appeared to block staining of all major proteins. Several cross-linked spots not previously seen on gels silver-stained for protein were observed at lower apparent molecular weights than the protein F oligomers seen in Fig. 3 and 5A. It thus appeared that certain species of LPS could also be cross-linked by DSP (Fig. 5D), either to other LPS molecules or to proteins. By vertically aligning the cross-linked products of gels stained for protein and LPS we were able to examine whether any of the protein F spots seen in Fig. 5A were due to LPS-protein F cross-linking. At least one spot due to LPS-protein F interaction (labeled FL) was seen in this way, but none of the protein F multimer spots noted above could be accounted for by protein F-LPS cross-links. It should be noted that both the O antigen and the rough core of LPS of *P. aeruginosa* contain amino groups (18, 19).

Two-dimensional analysis of uncross-linked outer membrane preparations was done as a control to reveal any disulfide-linked multimers (Fig. 5C). Only one high-molecular-weight protein was observed which consistently appeared below the diagonal. Although the identity of this

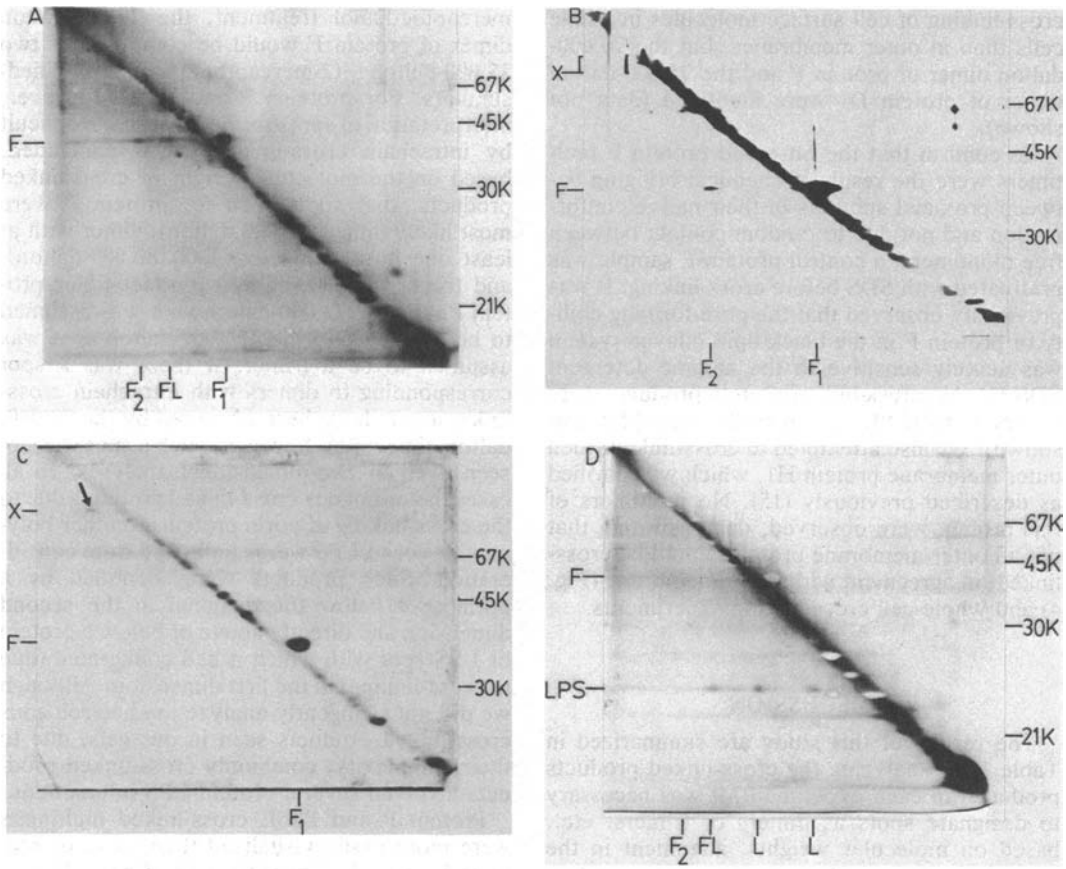

FIG. 5. Two-dimensional cross-linking analysis of outer membrane samples. Molecular weights of cross-linked and monomeric spots are presented in Table 1 and were determined with the molecular weight standards bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (21,000) obtained from Sigma Chemical Co., St. Louis, Mo. (A) Outer membranes were cross-linked with 200 μg of DSP per mg of protein for 30 s (note that the treatment time and consequently the extent of cross-linking is lower in this gel compared with the equivalent lane in Fig. 4) and run on a 12.5% polyacrylamide gel; the lane was cut out and soaked in reduction mix containing 2-mercaptoethanol to break DSP cross-links; and then the lane was laid on top of another 12.5% acrylamide running gel and run in the second dimension as described in the text. Cross-linked products revealed and labeled included a dimer of protein F (F_2) and a proposed cross-link between protein F and a particular species of LPS (FL; see also D), as well as higher-molecular-weight material unable to enter the first-dimension gel. The monomer of F (F_1) was 2-mercaptoethanol modifiable (9), accounting for its slightly off-diagonal position. (B) Outer membranes were treated as in (A) except that 40 μg of DSP per mg of protein and an 11% polyacrylamide gel were used. The lowered cross-linker concentration produced only one product, a dimer of F (F_2). An unknown, high-molecular-weight protein (X) was 2-mercaptoethanol modified but not cross-linked (see also C). (C) Uncross-linked control outer membranes were run on an 11% polyacrylamide gel as in (A). No visible off-diagonal spots other than protein X (possibly a disulfide-bonded dimer cleaved by 2-mercaptoethanol treatment) were observed. (D) Outer membranes were treated as in (A) except that the gel was stained for LPS (see text). Cross-linked products which appeared to be due to LPS-LPS interaction (L) as well as one proposed LPS-protein F interaction (FL) were observed. The spot labeled FL was identified by its vertical alignment with a protein F spot which did not stain by this procedure but is clearly seen in (A) stained for protein. The position of the dimer of protein F (F_2) is marked.

protein was not determined, it is likely that it forms an interchain disulfide-linked dimer which was cleaved by 2-mercaptoethanol between the first and second dimensions. No other disulfide-linked or SDS-resistant multimers of outer membrane proteins were detected.

Whole *P. aeruginosa* cells were cross-linked to further confirm that the cross-linked products seen in outer membrane experiments were actually native structures and not the result of rearrangement occurring during outer membrane isolation. The results showed more extensive

cross-linking of cell surface molecules in whole cells than in outer membranes, but the 70,000-dalton dimer of protein F and the 95,000-dalton dimer of protein D1 were identified (data not shown).

To confirm that the observed protein F multimers were the result of chemical bridging between proximal subunits in their native conformation and not due to random contact between free monomers, a control protein F sample was pretreated with SDS before cross-linking. It was previously observed that the pore-forming ability of protein F in the black lipid bilayer system was acutely sensitive to the anionic detergent SDS (3). No cross-linked protein products were observed in the SDS-pretreated control (data not shown). We also attempted to cross-link purified outer membrane protein H1, which was purified as described previously (15). No multimers of this protein were observed, demonstrating that not all outer membrane proteins could be cross-linked, in agreement with outer membrane (Fig. 4) and whole-cell cross-linking experiments.

DISCUSSION

The results of this study are summarized in Table 1. In analyzing the cross-linked products produced in each experiment, it was necessary to designate spots as dimers or trimers, etc., based on molecular weights, alignment in the second dimension with monomers of the protein of interest, and frequency of appearance (intensity of staining). For instance, one would expect multimers requiring only one cross-linking molecule to appear more frequently than multimers requiring two cross-linking molecules; therefore, dimers would appear much more frequently than trimers, even if the native structure were a trimer. The exact frequency of each species cannot be predicted in the absence of specific information about the number of amino groups involved in cross-linking reactions. For proteins F and D1, the molecular weight and frequency of appearance of cross-linked products of both were consistent with dimer and trimer forms. Since the molecular weights of the cross-linked products were measured in the absence of 2-mercaptoethanol treatment, the 70,000-dalton dimer of protein F would be composed of two 35,000-dalton (2-mercaptoethanol-unmodified) subunits. For proteins P and PhoE, however, interpretation of spots formed was made difficult by intrachain cross-links. It was concluded, based on the molecular weight of cross-linked products, that spots seen for protein P were most likely dimer (90,000-dalton), dimer with at least one intrachain cross-link (80,000-dalton), and trimer (145,000-dalton) products. For protein PhoE, the 73,000-dalton spot was assumed to be a dimer and the 100,000-dalton spot was assumed to be a trimer. If there was a spot corresponding to dimers with intrachain cross-links, it may have been obscured by the 73,000-dalton dimer spot; however, such a spot was not seen even in two-dimensional analyses. In all cases, heterologous cross-linked products due to the cross-linking of porin proteins to other polypeptides or to LPS were eliminated from consideration. Such products were identified by a porin spot below the diagonal in the second dimension and directly above or below a protein or LPS spot with which it had comigrated (due to cross-linking) in the first dimension. Although we did not stringently analyze the heterologous cross-linked products seen in our gels, due to their complexity, commonly cross-linked products involved several protein-LPS interactions.

Protein P and PhoE cross-linked multimers were more easily visualized than those of proteins F and D1. Since the ease of cross-linking reflects the availability of two cross-linker-reactive amino groups within 1.2 nm, one on each subunit, it may be that such amino groups are more accessible on both proteins P and PhoE. The similar cross-linking patterns of these proteins, despite their differences in molecular weight, may reflect their analogous functions in phosphate uptake through the outer membrane (14, 17). It has been shown (R. Benz, M. Gimple, K. Poole, R. Darveau, and R. E. W. Hancock manuscripts in preparations) that the selectivity filters (presumably the mouths of porin channels) of proteins P and PhoE have amino groups which appear to be important in their function as anion-selective channels. Acetylation of these groups partly neutralized the anion-

TABLE 1. Summary of the cross-linked multimers of porin proteins observed in this study

Protein	Assignment and mol wt				Native SDS-resistant oligomer
	Monomer	Dimer	Trimer	Other	
P. aeruginosa protein F	38,000	70,000	120,000	35,000 (minus 2-mercaptoethanol)	No
P. aeruginosa protein D1	47,000	95,000	145,000	29,000 (unheated)	No
P. aeruginosa protein P	48,000	90,000	145,000	44,000 (internal cross-link) 80,000	Yes
E. coli protein PhoE	38,000	73,000	100,000	34,000 (internal cross-link)	Yes

ic selectivity of the channels in black lipid bilayers but, at least for the PhoE protein, did not change the apparent size of the pore. Given the above-mentioned similarities between proteins P of *P. aeruginosa* and PhoE of *E. coli*, perhaps these two proteins are evolutionarily related. The difference in pore size could be explained if some of the extra 10,000-dalton portion of the larger protein P was present toward the interior of the channel. This heterogeneity might also explain the fact that protein P more easily yielded dimeric associations with internal cross-links whereas the PhoE protein cross-linked more readily to trimers.

The cross-linked bands of protein F were harder to visualize than those of proteins P and PhoE. This was primarily due to the fact that protein F did not result in distinct bands of cross-linked polypeptides in the first dimension even when the cross-linker concentration was varied over a 500-fold range (Fig. 3), using either whole outer membranes or purified protein F. Similar difficulties can be observed from examination of cross-linking patterns of the *Chromatium vinosum* 42,000-dalton protein (20). Analysis of the products of protein F cross-linking after cleavage and electrophoresis in the second dimension demonstrated that the dimer and trimer cross-linked products formed smeared spots which covered regions of the gels corresponding to about 60,000 to 80,000 daltons for the dimer and about 110,000 to 130,000 daltons for the trimer. These spots were not artifacts since the dimer was observed with low concentrations of cross-linker and also by using [^{35}S]methionine, Coomassie blue, or silver stain. Furthermore, the products in these molecular-weight regions corresponded to homologous cross-linked products rather than heterologous products since they did not vertically align with other polypeptide or LPS spots. Although the spots were spread over a range of molecular weights in the second dimension (as seen for other cross-linked porin oligomers [21, 32]), we estimated their molecular weights by using either limiting amounts of cross-linker, such that smaller spots were seen (e.g., Fig. 5B), or taking the center of the spots as the apparent relative mobility of the multimers. By either method of analysis, the molecular weights of the multimers were two (for the dimer)- and three (for the trimer)-fold multiples of the monomeric protein F molecular weight. Although the amount of trimer species for protein F (and for protein P; see Fig. 2) was considerably less than seen for the dimer species, it should be noted that, despite the fact that the lambda receptor (maltose porin) of *E. coli* is recognized to be a trimer, cross-linking with DSP results only in dimeric aggregates, with no trimer species demonstrated (33). This can be simply understood since chemical cross-linking will occur only if properly oriented amino groups spaced at 1.2-nm distance are available on adjacent subunits. Thus, if each monomer has only a single such group, the protein can never be cross-linked to a trimer (since a dimeric product would tie up two of the three accessible amino groups in the oligomer). Similarly, the probability of seeing a trimer will increase as the number of accessible, properly oriented amino groups increases. The smearing of the dimer and trimer bands in the first dimension could have a variety of explanations, including: (i) noncovalent interaction of protein F with LPS which forms a variety of bands in this area of the gel (19), (ii) intrinsic physical heterogeneity of protein F molecules since functional heterogeneity has been demonstrated (3, 25), (iii) differential monofunctional substitution of amino groups which are accessible to cross-linker but are improperly oriented for cross-linking, and (iv) the fact that protein F is known to be able to occupy at least six different molecular-weight positions on SDS-polyacrylamide gels according to temperature of solubilization and 2-mercaptoethanol concentrations (10).

Although we cannot definitively conclude that any of the proteins studied here form a specific trimer, we have clearly demonstrated that these proteins are oligomers, at least dimers, in their native states. By analogy with other porins in other organisms (21, 29, 30, 33), we feel that the most likely structure is a trimer and our results, although not proving this, are at least consistent with this proposal. If this is so, it is interesting that the trimeric form may be characteristic of all porins examined so far despite differences in channel size, selectivity, and physicochemical properties.

After this paper was submitted for publication, a paper by Yoshimura et al. was published (41) suggesting that protein F monomers were the functional form of this protein. Part of the evidence for protein F being in the monomeric form was a one-dimensional cross-linking analysis of SDS-treated protein F. Whereas these data agree with our data, it should be noted that we have recently demonstrated that protein F, even after treatment with SDS, contains some oligomeric products which can only be visualized by using Western immunoblots with monoclonal antibodies to protein F (Mutharia and Hancock, submitted for publication). This small level of trimers after SDS treatment is consistent with the low proportion of functional protein F channels (<1%) observed in in vivo assays (25).

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