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Large-scale purification and biochemical characterization of crystallization-grade porin protein P from *Pseudomonas aeruginosa*

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A large-scale purification scheme was developed for lipopolysaccharide-free protein P, the phosphate-starvation-inducible outer-membrane porin from *Pseudomonas aeruginosa*. This highly purified protein P was used to successfully form hexagonal crystals in the presence of *n*-octyl-β-glucopyranoside. Amino-acid analysis indicated that protein P had a similar composition to other bacterial outer membrane proteins, containing a high percentage (50%) of hydrophilic residues. The amino-terminal sequence of this protein, although not homologous to either outer membrane protein, PhoE or OmpF, of *Escherichia coli*, was found to have an analogous protein-folding pattern. Protein P in the native trimer form was capable of maintaining a stable functional trimer after proteinase cleavage. This suggested the existence of a strongly associated tertiary and quaternary structure. Circular dichroism studies confirmed these results in that a large proportion of the protein structure was determined to be β-sheet and resistant to acid pH and heating in 0.1% sodium dodecyl sulphate.

Introduction

The outer membranes of Gram-negative bacteria constitute molecular sieving permeability barriers. Permeation through the outer membrane barrier is mediated by a class of proteins termed porins which form transmembrane, water-filled channels with weak ion selectivity [1]. Porins generally allow the permeation of small hydrophilic molecules (below the exclusion limit of the channel) via a simple diffusion mechanism [1]. Protein P is an exception to this in that it forms phosphate-selective [2], anion-specific [3] channels, consistent with its demonstrated role in the phosphate-starvation-inducible, high-affinity phosphate transport system of *P. aeruginosa* [4]. Of the other porins studied to date, only the maltodextrin-selective LamB porin [5] and the polyphosphate-selective PhoE porin [6] have been shown to demonstrate substrate selectivity. Nevertheless, it has been proposed that the channels of LamB [5] and PhoE [7] are fundamentally different from that of protein P. For this reason, we have embarked upon an investigation of the protein chemistry of the P-porin to see if it possesses unique features that will help explain its functional uniqueness.

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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To date, only minimal information has appeared on the biochemistry of protein P. It has an apparent monomer molecular weight of 48000 and appears as an oligomer on sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis after solubilization in 2% SDS at temperatures less than 60 °C [8]. The oligomeric form appears to be a trimer, based on chemical crosslinking studies [9]. Antibody specific for the trimer does not interact with the monomer and vice versa [7], suggesting that there are no major linear epitopes present in the trimer form of the protein.

In this paper, we report on the large-scale purification of functional, lipopolysaccharide-free protein P oligomers and present data showing that these protein P oligomers are amenable to crystallization and a variety of structural studies.

Materials and Methods

Purification of protein P

Protein P was purified by a modification of the procedure previously described with alterations for large-scale preparation [2]. An 80 liters culture of *P. aeruginosa* strain AK1012 (a rough LPS derivative of strain PA01) was grown at 30 °C under phosphate-deficient conditions to an $A_{600} = 0.8$. The cells were harvested in a Sharples (Pennwalt Corp. Warminster, PA) centrifuge, followed by suspension in 5 mM MgCl, 10 mM Tris-HCl (pH 8.0) containing 50 μg/ml DNAase 1. The cell suspension was passed three times through a French pressure cell at 16000 psi. After unbroken cells were removed by low-speed centrifugation ($7000 \times g$, 5 min), cell envelopes were pelleted by ultracentrifugation at $180000 \times g$ for 1 h. The envelopes were suspended by extensive sonication (30 s at the highest setting of a Fisher Scientific (Vancouver, Canada) sonic dismembranator Model 300) in 2% (v/v) Triton X-100, 10 mM Tris-HCl (pH 8.0) at a total protein concentration of 10 mg/ml, followed by centrifugation at $100000 \times g$ for 1 h. The supernatant was discarded and the pellet resuspended in 2% (w/v) sodium dodecyl sulphate (SDS), 10 mM Tris-HCl (pH 8.0) followed by centrifugation as above. The final pellet was resuspended 2% SDS, 20 mM EDTA, in 10 mM Tris-HCl (pH 8.0) and centrifuged again. The resulting supernatant was applied to a Sephacryl S-200 superfine Pharmacia Canada, Dorval, Quebec) gel-exclusion column (90×2.5) equilibrated with 2% SDS, 10 mM Tris (pH 8.0), 1 mM EDTA, 3 mM NaN_3. The column effluent was monitored by following the A_{280} using a Pharmacia single-path ultraviolet monitor. 2-ml fractions were collected and A_{280} absorbing peaks were analyzed for protein content via SDS-polyacrylamide gel electrophoresis according to Hancock and Carey [10], using an 11% acrylamide-containing gel. Fractions containing protein P were pooled and run on 3-mm thick SDS slab gels using the Bio-Rad (Bio-Rad Laboratories, Mississauga, Ontario, Canada) Protean gel apparatus. After running, guide strips of the SDS-polyacrylamide gel were cut, stained with Coomassie brilliant blue and destained [12] to localize the protein P band. The appropriate area was cut out of the remaining gel and protein P passively eluted by soaking the strip in either 1% (w/v) *n*-octyl-β-D-glucopyranoside, 20 mM sodium phosphate (pH 7.0), 10 mM NaCl, 3 mM NaN_3, 3 mM dithiothreitol for crystallization trials or deionized H_2O for other purposes. To remove SDS for crystallization trials or amino acid analysis, the protein was extensively dialyzed against the same buffer. For protein sequencing the sample was extensively dialyzed against 0.1% SDS to remove residual salts. The purity of the protein P preparations was determined by SDS-polyacrylamide gel electrophoresis [10], using gels stained for protein with Coomassie brilliant blue or for lipopolysaccharide by the periodate-silver staining method of Tsai and Frasch [11] as described previously [12].

Protein P crystallization

The hanging-drop vapour-diffusion method of crystallization was used [13]. 5-μl drops were suspended from the underside of siliconized (Sigmacote; Sigma CHemicals, St. Louis, MO) glass coverslips over 1 ml reservoirs (in 24-well tissue-culture plates containing 15–35% (w/v) PEG 2000 in 40 mM NaCl, 40 mM Na_2HPO_4, 0.1% (w/v) NaN_3. The hanging drops contained approx. 4 μg of purified protein P, 0.8% (w/v) *n*-octyl-β-D-glucopyranoside, 20 mM Na_2HPO_4, 20 mM NaCl, 0.01% (w/v) NaN_3 and from 4% (w/v) to 34% (w/v) poly(ethylene glycol) 2000 (PEG 2000) at

pH values ranging from 4.0 to 7.0. Visible crystals were formed in 4–6% (w/v) PEG 2000 (initial concentration) equilibrated against reservoirs containing 15% (w/v) PEG 2000 at pH 4.0. A number of crystals were collected by low-speed centrifugation and resolubilized for SDS-polyacrylamide gel electrophoresis to check protein stability and purity.

Amino-acid analysis

Approx. 10-nmol portions of protein P (extensively dialyzed versus triple deionized water followed by acetone precipitation) were hydrolyzed in constant boiling 6 M HCl for 24 h. Phenol (1% w/v) was included to minimize degradation of tyrosine residues. The analyses were performed on a Beckman 119-CL automated amino-acid analyzer by S. Kielland, University of Victoria, Victoria, British Columbia, Canada.

Protein sequencing

Approx. 10 nmol of protein P extensively dialyzed versus 0.1% SDS in triple deionized water was subjected to 35 cycles of automated amino-terminal sequencing using the Beckman 890-C spinning cup sequencer by S. Kielland, University of Victoria, Victoria, British Columbia, Canada.

Proteinase digestion of protein P

Purified protein P trimer was enzymatically digested with the following enzymes: (1) TPCK-trypsin (Sigma) in 20 mM Tris-HCl (pH 8.0); (2) *Staphylococcus aureus* V8 proteinase (Sigma) in 20 mM Tris-HCl (pH 8.0) containing 35 mM $MgCl_2$; (3) papain (papainase Type 4, Sigma) in 2 mM EDTA, 20 mM Tris-HCl (pH 6.0); (4) proteinase K (Sigma) in 20 mM Tris-HCl (pH 8.0), for 2 h at 60 °C; (5) pronase (proteinase type IV, Sigma) in 20 mM Tris-HCl (pH 8.0); and (6) chymotrypsin (Sigma) in 20 mM Tris-HCl (pH 8.0). In all cases the enzyme/protein ratio was 1 : 100. Except where indicated, all digestions proceeded for 2–24 h at 37 °C. The extent of digestion was monitored by SDS gel electrophoresis [10].

To determine whether any cell-surface proteinase sensitive sites of protein P existed, whole cells grown in phosphate-limited media were subjected to proteolysis with the above enzymes as described elsewhere [14].

Black lipid bilayer experiments

Single channel conductance studies were carried out as previously described [6] using 1% oxidized cholesterol to form the supporting lipid bilayer. Small aliquots of purified protein P or proteinase-digested protein P (10^{-3} M) were added to the solution bathing the bilayers (1 M KCl) and the stepwise increase in conductance was recorded. A test of the functional stability of protein P was done by using both protein P samples prepared for crystallization and protein P which had been crystallized and then resolubilized in *n*-octyl-β-D-glucopyranoside containing Hepes buffer (0.05 M, pH 7.0).

Circular dichroism measurements

These measurements were made on a Jasco 500C spectropolarimeter interfaced with a DP500N data processor. The cell was maintained at 25 °C using a Lauda RM6 circulatory water bath. Near-ultraviolet (320–250 nm) scans were performed in a microcell, with a pathlength of 1 cm, which required only 90 μl of solution. The concentration of protein ranged from 1.0–1.5 mg/ml. Far-ultraviolet (250–184 nm) scans were done in a 0.0103 cm pathlength cell. The protein concentration varied from 0.3 to 0.5 mg/ml. In all instances, absorbances were kept below 1.5 (600 V on the photomultipler tube). The computer-averaged trace of either four or eight scans was employed in all calculations. Signal due to the solvent (10 mM Tris-HCl (pH 8.0), 0.1% SDS) was subtracted. The instrument was routinely calibrated with *d*(+)-10-camphorsulfonic acid at 290 nm and pantoyl lactone at 219 nm, as outlined by the manufacturer. The mean residue ellipticity $[\theta]$, expressed in $\text{deg} \cdot \text{cm}^2 \cdot \text{decimole}^{-1}$, was calculated on the basis of a mean residue molecular weight of 115.

Concentrations of protein P were determined by the absorbance at 278 nm with an absorption coefficient of 0.743, using the refraction method of Babul and Stellwagen [15] in which the absorbance of a protein sample is correlated with its weight concentration as determined from synthetic boundary experiments in the analytical centrifuge. An average refraction increment of 4.1 fringes/mg per ml was used in these calculations. Prior to these measurements, protein P samples

were dialyzed for 24 h against 500 Vol. of 0.1% SDS, 10 mM Tris-HCl (pH 8.0) to ensure complete equilibration.

In addition to spectral plots of mean residue weight ellipticity versus wavelength, secondary structure was determined using the computer program CONTIN developed by Provencher and Glöckner [16] which analyzes CD spectra as a sum of the spectra of 16 proteins where structures are known from X-ray crystallography. Program input was mean residue weight ellipticities in 1 nm intervals from the minimum value measured to 240 nm.

Results

Purification and crystallization

Due to the large quantity of protein and high degree of purity necessary for crystallization, a large-scale isolation and purification protocol was developed for protein P. From 105 g wet weight of cells, approx. 2.5 g of crude extract was obtained by detergent solubilization. Further purification via gel filtration, elution from SDS-polyacrylamide gels and dialysis resulted in a final yield of approx. 500 mg of pure protein P. Purity was monitored by SDS-polyacrylamide gel electrophoresis staining either with Coomassie brilliant blue [10] to detect protein contaminants or silver staining after periodate treatment [11] for the detection of lipopolysaccharide. Fig. 1 shows preparations of protein P at various stages in the protocol. The final preparation was lipopolysaccharide-free and protein P appeared as a single, tight band in both the trimer (unheated in SDS) and monomer (boiled in SDS) form. We also examined protein P which had been crystallized prior to loading on the gel (Fig. 1, lanes 4 and 8). Very little degradation of protein P occurred even after sitting at room temperature for 4–6 months in crystallization buffer.

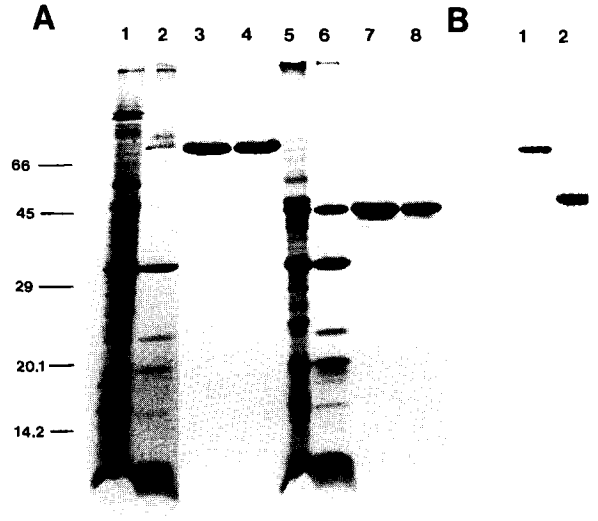

Fig. 1. SDS-polyacrylamide gel electrophoresis of protein P purification. (A) Lanes 1–4 were not heated prior to electrophoresis, lanes 5–8 were heated at 100 °C for 10 min. Stained with Coomassie brilliant blue. Lanes 1 and 5, cell envelope fraction; lanes 2 and 6, SDS-EDTA soluble fraction; lanes 3 and 7, purified protein P; and lanes 4 and 8, protein P resolubilized from crystallized state. (B) Silver stain of purified protein P showing no lipopolysaccharide contamination. Lane 1, unheated, and lane 2 heated at 100 °C for 10 min. Molecular weights of protein standards ($\times 10^3$) are indicated on the left.

Protein P crystals were obtained at pH 4.0 using PEG 2000 as the precipitant. Initial concentrations of 4–6% (w/v) PEG 2000 produced crystals of a hexagonal nature approx. 0.08 mm long $\times$ 0.05 mm in diameter. To date, no other crystal forms have been generated, although a range of conditions have been examined. Only protein samples completely free of lipopolysaccharide were found to form crystals. Protein P was analyzed in black lipid bilayer membranes to demonstrate retention of its pore-forming ability. A typical [8] single channel conductance of 0.25 nS in 1 M KCl was demonstrated for protein P both prior to and after crystallization.

Amino-acid analysis and N-terminal sequence

The amino-acid composition of protein P is listed in Table I along with the previously published compositions of the *P. aeruginosa* major porin protein F [17], the phosphate-starvation-inducible PhoE porin from *E. coli*, and a major *E. coli* porin, OmpF [18]. Unlike protein F, protein P had no cysteine residues. The amino-acid compositions appeared fairly similar between all four proteins, with each containing approx. 30% charged residues and with a reasonably large proportion (more than 40%) of the residues being hydrophobic. Similar compositions exist for other *P. aeruginosa* outer membrane proteins (E, I and H [17]). The amino-terminal amino-acid sequence

TABLE I

AMINO-ACID ANALYSES OF SELECTED *P. AERUGINOSA* AND *E. COLI* PORIN PROTEINS

The analyses for PhoE and OmpF are derived from the nucleotide sequences presented by Overbeeke et al. [18], and for protein F from the amino-acid analysis of Mizuno et al. [17]. n.d., not determined.

Amino acid	Composition (mol%)			
	P	F	PhoE	OmpF
Ala	10.4	9.5	8.1	8.5
Val	5.5	8.5	3.6	6.8
Leu	6.8	4.8	6.4	6.2
Ileu	11.8	2.4	3.9	3.5
Pro	1.6	3.1	0.9	1.2
Phe	3.6	3.7	6.4	5.6
Trp	n.d.	0.7	0.9	0.6
Met	1.3	1.7	2.1	0.9
Gly	14.3	11.2	10.9	14.4
Ser	8.3	6.4	5.4	4.7
Thr	6.8	5.4	7.0	6.2
half Cys	–	1.3	–	–
Tyr	5.2	5.4	6.7	8.5
Asx	14.8	15.3	17.6	16.7
Lys	4.9	4.3	7.0	5.3
Arg	5.7	4.2	3.6	3.2
Glx	8.0	10.2	9.1	7.3
His	0.8	1.8	0.3	0.3

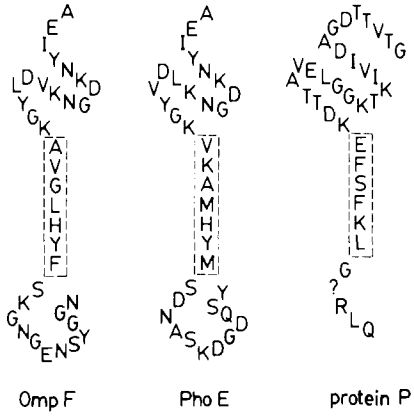

Fig. 2. Amino-terminal sequence and corresponding protein-folding pattern of protein P, and the *E. coli* porins PhoE and OmpF. The sequences for PhoE and OmpF are from Overbeeke et al. [18]. The deduced protein-folding patterns were obtained according to the prediction rules of Paul and Rosenbusch [19]. The residues enclosed by dotted lines are the probable membrane-spanning regions.

for proteins P, PhoE and OmpF, presented as the deduced secondary structure folding patterns determined by the method of Paul and Rosenbusch [19], are shown in Fig. 2. This is a protein-folding prediction method designed for membrane proteins which lack predominant hydrophobic domains and have a high percentage of β-pleated sheet structure. Automated sequencing of protein P produced the first 36 residues of the native protein. Although little sequence homology was apparent between protein P and the other two porins (in contrast, a high percentage of homology is apparent between the two *E. coli* porins [18]), a distinct similarity existed in the folding patterns of the N-terminal portion of these three membrane-spanning, pore-forming molecules.

Proteolysis of protein P

Protein P in the trimer-form was found to be resistant to several proteinases. The proteolytic enzymes, chymotrypsin (Fig. 3), papain and *S. aureus* proteinase (data not shown), did not digest protein P trimers, as was detected by SDS-polyacrylamide gel electrophoresis. However, the enzymes trypsin (Fig. 3), pronase and proteinase K (data not shown) cleaved protein P to a lower apparent weight (approx. 37000 as compared to 47000 for native protein P), as detected by a change in mobility of the heat-denatured monomers on SDS-polyacrylamide gels (Fig. 3, lanes 4–6). However, if the proteinase-treated protein P was not denatured into monomers prior to electrophoresis, no alteration in these proteinase-treated protein P trimers was observed (Fig. 3, lanes 1–3). This suggested that the cleaved sequences remained associated with the trimer as a result of tertiary or quarternary structure interactions.

To determine if the proteinase-sensitive sites of protein P observed after digestion with trypsin, pronase or proteinase K were cell-surface located, intact bacteria were subjected to proteolysis, then analyzed by SDS-polyacrylamide electrophoresis. No proteolysis was observed (data not shown) indicating that the proteinase-sensitive sites were either buried in the outer membrane or located on the inner surface of the outer membrane.

To assess the effect of proteinase-treatment on the pore-forming function of protein P, trypsin-, pronase- and proteinase K-digested samples of the

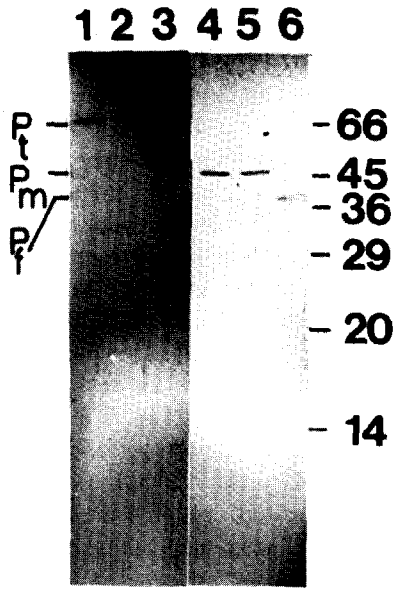

Fig. 3. SDS-polyacrylamide gel electrophoretograms of native and proteinase-treated protein P trimer. P_t = P trimer, P_m = P monomer, P_f = proteolytic fragment of P. Samples (trimer forms) in lanes 1–3 were not heated in SDS prior to electrophoresis, whereas the samples (monomer forms) in lanes 4–6 were heated at 100 °C for 10 min prior to electrophoresis. Lanes 1 and 4, untreated protein P; lanes 2 and 5, protein P digested with chymotrypsin; and lanes 3 and 6, protein P digested with trypsin. Molecular weights of protein standards ($\times 10^3$) are indicated on the right.

protein P trimer were analyzed by the black lipid model membrane system. In all three cases, the digested protein P formed channels with the same conductance as undigested protein P (0.25 nS in 1 M KCl), indicating that this digestion has no effect on the function of this protein.

Circular dichroism studies

Far-ultraviolet CD data for protein P are presented in Fig. 4. The spectrum of the native protein in 0.1% SDS, 10 mM Tris-HCl (pH 8.0) showed a broad minimum centred at 212 nm, $[\theta]_{212} = -3310°$, with a peak at 189 nm, $[\theta]_{189} = 6410°$. Boiling a protein P solution for 10 min produced a reduction in negative ellipticity, particularly around 205 nm, $[\theta]_{205} = -5280°$. The positive peak at 189 nm was also somewhat reduced, $[\theta]_{189} = 4270°$. The helix-forming solution, 0.82% SDS at pH 2.0, had surprisingly little in-

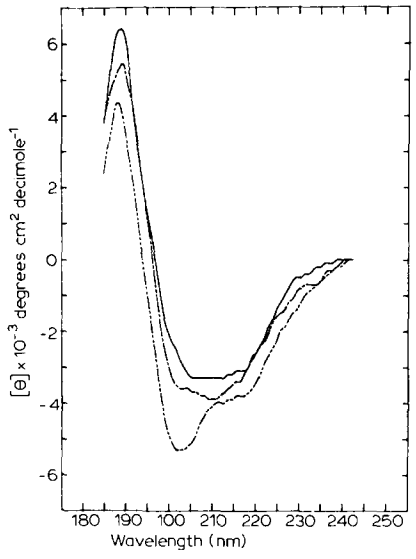

Fig. 4. Far-ultraviolet CD spectra for protein P from *P. aeruginosa*. The solvent was 0.1% SDS, 10 mM Tris-HCl (pH 8.0). The protein concentration was 0.2 mg/ml and the spectra were recorded at 25 °C. Symbols: native protein (———), protein boiled for 10 min (- - — - - -), and protein treated with 0.82% SDS at pH 2.0 (- — - —-).

fluence on the CD spectrum attesting to the stability of protein P structure at acid pH. The near-ultraviolet CD spectrum is shown in Fig. 5. There was a weak negative trough near 310 nm, a sharp

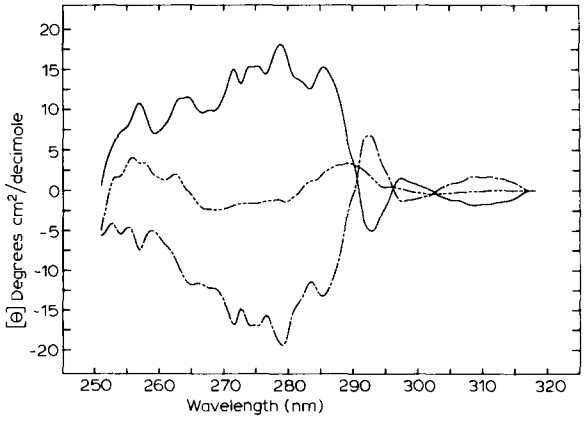

Fig. 5. Near-ultraviolet CD spectra for protein P from *P. aeruginosa*. The solvent was 0.1% SDS, 10 mM Tris-HCl (pH 8.0). Protein concentration was 1.01 mg/ml and the spectra were obtained at 25 °C using a 1 cm pathlength. Symbols: native protein (———), boiled for 15 min (- - — - -), and a difference spectrum between native and boiled forms (- — - — -).

minimum at 293 nm and the remainder of the spectrum was essentially a broad positive peak with fine-structure effects superimposed on it. After boiling the sample, the weak trough at 310 nm was eliminated along with the minimum at 293 nm, whilst the fine-structured and positive nature of the spectrum below 290 nm was greatly reduced.

The far-ultraviolet CD data were analyzed by the computer program CONTIN of Prevencher and Glöckner [16] to estimate the amount of α-helix, β-sheet, β-turn and aperiodic structure present in protein P. The results are presented in Table II. Native protein P had 3% α-helix, 65% β-pleated sheet, 26% β-turn, and 6% aperiodic formation in its structure. Boiling a solution of protein P in 0.1% SDS for 10 min induced an increase in the amount of α-helix and random coil presumably at the expense of β-sheet structural elements. Acidic SDS, a helix promoter, produced essentially no alteration in structure. This was consistent with our previous observations (Worobec, E., unpublished data) that protein P was resistant to conditions of low pH (pH = 2.0) as determined by the lack of change in the mobility of this protein on SDS-polyacrylamide gels.

Discussion

The biochemical characterizations of protein P presented in this communication warranted the development of a large-scale purification scheme. All the procedures used required highly pure protein, completely free of outer membrane lipopolysaccharide. We were successful in obtaining a large amount of lipopolysaccharide-free protein P by using as a final step, the elution of the protein from SDS gels. *P. aeruginosa*, O-antigen-containing lipopolysaccharide comigrates on SDS-polyacryalmide gels with protein P. Therefore we isolated protein P from envelopes of the phosphate-starved *P. aeruginosa* strain AK1012, an O-antigen-deficient mutant strain of our laboratory wild-type strain PA01. One advantage of our scheme for purifying crystallization-grade protein P over published methods for purifying lipopolysaccharide-free *E. coli* OmpF porin is that our protein retained channel function in black lipid bilayer membranes, wheras the OmpF porin was apparently inactive [20]. Nevertheless, it is interesting that the hexagonal form of protein P crystals observed by us was one of the crystal forms obtained by Garavito et al. [13] for the OmpF porin.

In this study, we have demonstrated that protein P had gross functional and structural similarities to other studied porin proteins. Poole and Hancock [7] revealed that a distinct cross-reaction existed between protein P and many phosphate-starvation-inducible porins from bacteria in the Enterobacteriaceae and the Pseudomonadaceae, including PhoE from *E. coli*. However, this cross-reaction occurred only between the native protein forms (trimers) and not between heat-denatured monomers. As this antibody cross-reactivity appears to be based on a conformational determinant involving the tertiary structure of these proteins, it was not surprising that no homology existed at the primary amino-acid level between the amino-termini of protein P and the two *E. coli* porins, but that a distinct similarity existed in the overall structure of the amino-termini of these proteins.

Circular dichroism studies of protein P revealed that it had a large proportion of β-sheet structure in the trimer form as observed for other porins [1]. The β-sheet structure was substantially resistant to treatment at acid pH in 0.82% SDS and to treatment at 100 °C in 0.1% SDS (Table II). Nevertheless, it should be noted that treatment at 100 °C in 2% SDS caused complete denaturation of the protein (Fig. 3, lane 4). The apparent lack of linear epitopes in the trimer [7], as well as the resistance of trimers to digestion by some proteinases and

TABLE II

STRUCTURE ANALYSIS OF *P. AERUGINOSA* PROTEIN P AS DETERMINED FROM FAR-ULTRAVIOLET CD DATA ANALYZED ACCORDING TO PROVENCHER AND GLÖCKNER [16]

Condition	Structure parameter			
	α-helix	β-sheet	β-turn	remainder
0.1% SDS	0.03	0.65	0.26	0.06
0.1% SDS, 100 °C, 10 min	0.08	0.55	0.25	0.12
0.82% SDS (pH 2.0)	0.04	0.66	0.27	0.04

the maintenance of a stable, functional protein after digestion by other proteinases (Fig. 3) supported the concept that protein P had a strongly associated tertiary and quaternary structure, in confirmation of the far-ultraviolet CD spectral data.

The near-ultraviolet CD spectra of proteins in general, originates from the aromatic amino acids, tyrosine, phenylalanine and tryptophan, being located near asymmetric optically-active centre. Protein P showed a fairly complicated spectrum, indicative of contributions from all types of aromatic amino acids. The change from positive to negative ellipticity noted when protein P was boiled in 0.1% SDS suggested changes in the enviroments around key aromatic residues, these being the bulkiest residues in the protein. Thus, protein folding might have changed to allow an interchange of aromatic residues into new optically active centres or this data may have reflected the partial or complete disaggregation of the trimer to the monomer form. Since the far-ultraviolet spectra indicated that there was still a significant amount of β-sheet conformation present, it would appear that extensive changes to the tertiary structure of the protein did not occur upon boiling. This again emphasized the structural stability of protein P.

In a recent review, the hypothesized structures for the major porins studied to date was presented [21]. Electron diffraction studies of lipid bilayers containing the *E. coli* porin OmpF have shown that the functional trimer contains three separate openings at the surface which join to form a single opening near the centre of the membrane [22]. On the other hand, the membrane-spanning structures of PhoE of *E. coli* and protein F of *P. aeruginosa* have been speculated to consist of three separate channels, not joined as with OmpF [21]. We predict a gross similarity in the structure of protein P as compared to these outer membrane porins; however, it is expected that the major differences will lie in the nature of the channel, with the protein P channel containing a narrow constriction, as determined by our black lipid bilayer studies [2,3]. This constriction apparently contains the phosphate-binding site which is proposed to involve three symmetrically located lysine residues, one from each of the monomer subunits of the functional trimer [2]. It is interesting to note that one quarter of the lysines were found in the first 35 residues, two of which were contained in the predicted membrane-spanning region, and one of which may be contributing to the channel constriction.

Further analyses of protein P using crystallographic methods in conjunction with the determination of the complete amino-acid sequence (work in progress), will allow us to study the three-dimensional structure and mechanism of this phosphate-specific porin protein in more detail.

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References

1 Hancock, R.E.W. (1986) Bacterial Outer Membranes as Model Systems (Inouye, M., ed.), pp. 187–225, John Wiley & Sons, New York.
2 Hancock, R.E.W. and Benz, R. (1986) Biochim. Biophys. Acta 860, 699–707.
3 Benz, R., Gimple, M., Poole, K. and Hancock, R.E.W. (1983) Biochim. Biophys. Acta 730, 387–390.
4 Poole, K. and Hancock, R.E.W. (1986) Mol. Gen. Genet. 202, 403–409.
5 Benz, R., Schmid, A., Nakae, T. and Vos-Scheperkeuter, G.H. (1986) J. Bacteriol. 165, 978–986.
6 Dargent, B., Hofmann, W., Pattus, F. and Rosenbusch, J.P. (1986) EMBO J. 5, 773–778.
7 Poole, K. and Hancock, R.E.W. (1986) J. Bacteriol. 165, 987–993.
8 Hancock, R.E.W., Poole, K. and Benz, R. (1982) J. Bacteriol. 150, 730–738.
9 Angus, B.L. and Hancock, R.E.W. (1983) J. Bacteriol. 155, 1042–1051.
10 Hancock, R.E.W. and Carey, A.M. (1979) J. Bacteriol. 140, 902–910.

11 Tsai, C.M. and Frasch, C.E. (1982) Anal. Biochem. 119, 115–119.
12 Parr, T.R., Poole, K., Crockford, G.W.K. and Hancock, R.E.W. (1986) J. Bacteriol. 165, 523–526.
13 Garavito, R.M., Jenkins, J., Jansenius, J.N., Karlson, R. and Rosenbusch, J.P. (1983) J. Mol. Biol. 169, 313–327.
14 Mutharia, L.M. and Hancock, R.E.W. (1985) Can. J. Microbiol. 31, 381–386.
15 Babul, J. and Stellwagen, E. (1969) Anal. Biochem. 28, 216–221.
16 Provencher, S.W. and Glöckner, J. (1981) Biochemistry 20, 33–37.
17 Mizuno, T. and Kageyana, M. (1979) J. Biochem. 86, 979–989.
18 Overbeeke, N., Bergmans, H., VanMansfield, F. and Lugtenberg, B. (1983) J. Mol. Biol. 163, 513–532.
19 Paul, C. and Rosenbusch, J.P. (1985) EMBO J. 4, 1593–1597.
20 Schindler, H. and Rosenbusch, J.P. (1981) Proc. Natl. Acad. Sci. USA 78, 2302.
21 Hancock, R.E.W. (1987) J. Bacteriol. 169, 929–933.
22 Engel, A., Massalski, A., Schiveller, M., Dorset, D.L. and Rosenbusch, J.P. (1985) Nature (London) 317, 643–645.