***Burkholderia* (formerly *Pseudomonas*) *cepacia* porin is an oligomer composed of two component proteins**

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The 81 kDa protein (designated OpcPO) which forms a diffusion pore in the outer membrane of *Burkholderia* (formerly *Pseudomonas*) *cepacia* has a unique characteristic in that when the purified protein is heated it yields a major 36 kDa protein (designated OpcP1) and a minor 27 kDa protein (designated OpcP2). Moreover, incubation of OpcPO in citrate buffer at pH 3.0 produced an unusual dissociation into 72 kDa and 27 kDa proteins. For the characterization of OpcPO and its derivatives, OpcP1 and OpcP2 from purified OpcPO were isolated by preparative SDS-PAGE. Reconstitution of OpcPO using purified preparations of OpcP1 and OpcP2 indicated that these derivatives were not proteolytic fragments of OpcPO. Moreover, immunoblot assays with murine polyclonal antisera specific for OpcP1 and OpcP2 yielded the following results: (i) OpcP1 and OpcP2 are immunologically distinguishable proteins; (ii) the unusual dissociation of OpcPO in citrate buffer at pH 3.0 resulted in the release of OpcP2 from OpcPO, and the resulting 72 kDa protein was probably an oligomer of OpcP1; (iii) purified OpcP1 itself produced two additional 53 kDa and 72 kDa proteins spontaneously following elution from the bottom of the SDS-PAGE gel. From these findings, it was concluded that OpcPO is formed by the non-covalent association of OpcP2 with an oligomer of OpcP1 that has the ability to self-assemble.

Keywords: *Burkholderia* (*Pseudomonas*) *cepacia*, porins, outer membrane protein, self assembly

INTRODUCTION

Burkholderia (formerly *Pseudomonas*) *cepacia* is recognized as a pathogenic agent in nosocomial infections in immuno-compromised patients, and causes pulmonary infections in patients with cystic fibrosis (Philips & Eykyn, 1971; Ederer & Matsen, 1972; Gilligan, 1991; Smith *et al.*, 1993). One of the clinically important characteristics of *B. cepacia* is its intrinsic high resistance to structurally unrelated antimicrobial agents and disinfectants (Fass & Barnishan, 1980; Kropp *et al.*, 1985; Gilligan, 1991). Antimicrobial agents other than those affecting the membrane itself have to pass through the outer membranes of Gram-negative bacteria to reach their targets. Accordingly, the permeability of the outer

membrane to antimicrobial agents influences their effectiveness (Nikaido & Vaara, 1985; Nikaido, 1989). The intrinsic resistance of *B. cepacia* to β -lactam antibiotics has been demonstrated to be caused by the low permeability of the outer membrane, which is approximately 10 times less permeable than that of *Escherichia coli* and comparable to that of another highly resistant bacterium, *P. aeruginosa* (Parr *et al.*, 1987). Hydrophilic solutes such as most β -lactams diffuse via water-filled channels in the outer membrane, called porins (Nakae, 1975, 1976), to reach their targets (Nikaido & Rosenberg, 1983; Yoshimura & Nikaido, 1985; Nikaido & Vaara, 1985; Hancock, 1987). Therefore, the characteristics of the porin pore are significant determinants of susceptibility to β -lactams (Nikaido, 1989).

Analysis by SDS-PAGE determined that the apparent molecular masses of the major outer membrane proteins of *B. cepacia* cells grown in nutrient broth are 40 kDa,

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36 kDa, 24.5 kDa, 17 kDa and 14.5 kDa, and of these, the 36 kDa and the 24.5 kDa proteins are heat-modifiable (Anwar *et al.*, 1983). Several proteins of the major outer membrane proteins have recently been characterized as follows: (i) The major outer membrane protein of 81 kDa dissociates into a major 36 kDa protein and a minor 27 kDa protein (probably the same as the above 24.5 kDa protein) by heat solubilization and is able to form a diffusion pore in the black lipid bilayer system (Parr *et al.*, 1987). (ii) The function of the 81 kDa protein was suggested by the finding that high-level β -lactam resistance was associated with decreased expression of the 36 kDa and the 27 kDa proteins (Aronoff & Stern, 1988). (iii) The 40 kDa protein (OpcS) allows the penetration of chloramphenicol but not penicillin G in proteoliposome membranes (Burns & Clark, 1992). These findings indicate that the 81 kDa and the 40 kDa proteins act as porins in the *B. cepacia* outer membrane. Investigation of the function and structure of the structurally unique 81 kDa protein as compared with the ordinary porin oligomer in Gram-negative bacteria (Nikaido & Vaara, 1985; Nakae, 1986; Benz, 1988; Nikaido & Red, 1990; Rosenbusch, 1990) might be useful not only for a general understanding of the drug resistance of *B. cepacia*, but also for understanding how porins assemble. Despite little information regarding the relationship between the 36 kDa and the 27 kDa proteins, they seem to have been recently accepted as independent components (Aronoff, 1988) or subunits (Burns & Clark, 1992) from which the 81 kDa protein is constructed.

In this paper we found by observation of the dissociation of the 81 kDa protein and reassociation of its dissociated proteins, and by immunoblots with murine antiserum specific to each of the two dissociated proteins, that the 81 kDa protein is a hetero-oligomer composed of two immunologically distinct component proteins. Moreover, we propose new designations for these porin proteins.

METHODS

Purification of OpcPO. Parr *et al.* (1987) purified the 81 kDa porin oligomer (OpcPO) by gel filtration of proteins obtained from residues following SDS extraction of the outer membranes isolated by sucrose density gradient fractionation of *B. cepacia* whole cell lysates. This suggested that OpcPO is a peptidoglycan-associated protein. Therefore, to improve recovery, we used total membranes (cell envelopes) as our starting material instead of outer membranes, and further, used lithium dodecylsulfate (LDS) instead of SDS to prevent crystallization during chromatography.

Briefly, cells grown in L broth consisting of 1% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract (Difco) and 0.5% (w/v) NaCl were harvested by centrifugation at 10000 *g* for 30 min at 4 °C. The cells were resuspended in 10 mM Tris/HCl, pH 8.0, containing 0.1 mg each of DNase and RNase ml⁻¹, and were passed twice through a French pressure cell at 12000 p.f.i. After removing unbroken cells by centrifugation, total membranes were pelleted from the resulting supernatant by centrifugation at 200000 *g* for 1 h. Almost all the membrane proteins in the pellet were removed by extracting twice with 10 mM Tris/HCl, pH 8.0, containing 2% (w/v) LDS followed by centrifugation

at 200000 *g* for 1 h. Pelleted LDS-insoluble materials (crude peptidoglycan) were suspended in a small volume of 10 mM Tris/HCl, pH 8.0, containing 2% LDS and 0.5 M LiCl (Buffer A), and incubated at 37 °C for 1 h. This suspension was then centrifuged at 200000 *g* for 1 h at 37 °C and the supernatant was loaded onto a Sephacryl S-200 superfine gel (Pharmacia) column (2.4 × 90 cm) equilibrated with Buffer A and eluted with the same buffer. To remove LDS and LiCl, OpcPO-rich peaks were pooled and dialysed in 10 mM Tris/HCl, pH 8.0, for 2 d. Next, a non-ionic detergent, 2% (w/v) nonanoyl-*N*-methylglucamide (MEGA-9; Dojindo), was substituted for the LDS by gel filtration using a Sephadex G-25 gel (Pharmacia) column equilibrated with Buffer B consisting of 2% (w/v) MEGA-9 in 50 mM sodium phosphate buffer, pH 7.5. The OpcPO preparation was then loaded onto a Buffer B-equilibrated TSKgel CM-5PW column (0.75 × 7.5 cm; Tosoh) on a high performance liquid chromatograph and was fractionated by elution with a 0–0.5 M linear gradient of NaCl in Buffer B.

Gel purification of OpcP1 and OpcP2. Purification of OpcP1 and OpcP2 was performed with a preparative continuous-elution SDS-PAGE apparatus (Prep Cell Model 491; Bio-Rad) using the discontinuous buffer system reported by Laemmli (1970). Briefly, a preparation of the purified OpcPO was mixed with 5 vols 6 M guanidine hydrochloride solution. After incubation for 3 h at 37 °C, guanidine hydrochloride was removed by dialysis against pure water. The dialysed sample was mixed with the same volume of the SDS sample buffer (Hancock & Carey, 1979) consisting of 1 M Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.05% bromophenol blue, and applied to a stacking gel of 4% T, 2.67% C in the Prep Cell. Proteins which migrated off the bottom of the separating gel of 10% T, 2.67% C were collected from the flow of Buffer B and observed by an analytical SDS-PAGE.

Production of respective antisera. BALB/c female mice were immunized with 20 µg purified OpcP1 or OpcP2 on days 1, 7, 14 and 17. The first injected protein was in Freund's complete adjuvant (Difco), the second and the third were in Freund's incomplete adjuvant (Difco), and the fourth was in PBS. The first, the second and the third were administered intraperitoneally, and the fourth was injected intravenously. Antibody titres in the respective sera were measured by ELISA using crude outer membranes released from briefly sonicated *B. cepacia* as the antigen and peroxidase-conjugated antimouse immunoglobulins (IgG, IgM, IgA) goat IgG (Cappel) as the secondary antibodies according to the method of Harlow & Lane (1988). Antibody binding was detected using a peroxidase substrate kit (ABTS, Bio-Rad) and colour development was measured at 415 nm with a microplate reader.

Western immunoblot analysis. Proteins fractionated by SDS-PAGE were electrophoretically transferred to nitrocellulose paper (0.45 µm pore size; Bio-Rad) as described by Towbin *et al.* (1979). After blocking with PBS, pH 7.4, containing 5% (w/v) non-fat dry milk and 0.2% Tween 20, the blots were incubated at 37 °C for 1 h with the above described peroxidase-conjugated IgG. Peroxidase activity was visualized with a Horseradish Peroxidase Substrate Kit (Bio-Rad).

SDS-PAGE and molecular mass determination. Analytical SDS-PAGE was performed using the discontinuous buffer system described by Laemmli (1970) with a 10% T, 2.60% C separating gel. Samples for SDS-PAGE were diluted with an equal volume of SDS sample buffer, and protein bands were visualized by staining with Coomassie brilliant blue. The migration distance of individual proteins following SDS-PAGE

was determined by densitometry with an LKB Ultrosan XL, and molecular masses were estimated from a linear plot of log(mass) of standard markers versus their migration distances. Rabbit skeletal muscle myosin (200 000 Da), *E. coli* β -galactosidase (116 250 Da), rabbit muscle phosphorylase b (97 400 Da), bovine serum albumin (66 200 Da), hen egg white ovalbumin (45 000 Da), bovine carbonic anhydrase (31 000 Da), soy bean trypsin inhibitor (21 500 Da), and hen egg white lysozyme (14 400 Da) were used as standard markers.

Determination of protein. Protein was quantified by the Lowry method.

Purification of OmpF. Purification of the OmpF oligomer from *E. coli* B was carried out by the method described by Tokunaga *et al.* (1979).

RESULTS

Purification of OpcPO, OpcP1 and OpcP2

For the characterization of *B. cepacia* porin, the 81 kDa porin oligomer was purified from cells of strain ATCC 25416. Most proteins of the total membrane fraction (cell envelope) were solubilized by LDS (Fig. 1, lane 1). Incubation of the insoluble residues in the buffer containing LDS and LiCl released several protein species in the size range 81 kDa to 140 kDa (Fig. 1, lane 2). Heating of these proteins to 100 °C caused the appearance of two proteins with molecular masses 36 kDa and 27 kDa, respectively, and the disappearance of all 81 kDa–110 kDa

Fig. 1. Purification of the 81 kDa protein. Cell envelope proteins from *B. cepacia* ATCC 25416 were fractionated as described in Methods. Samples were solubilized at 25 °C for 10 min (lanes 2, 4 and 6) or at 100 °C for 10 min (lanes 1, 3, 5 and 7) and subjected to SDS-PAGE. Lanes: 1, LDS extracts (20 μ g protein) from envelopes; 2 and 3, proteins released from crude peptidoglycan (15 μ g protein); 4 and 5, an OpcPO-rich fraction (7 μ g protein) after fractionation by gel filtration chromatography with Sephacryl S-200; 6 and 7, an OpcPO fraction (7 μ g protein) after ion-exchange chromatography with TSKgel CM-5PW. Arrowheads show the 36 kDa (OpcP1) and the 27 kDa (OpcP2) proteins dissociated from the purified 81 kDa protein. The mobilities of standard proteins and apparent molecular masses of relevant protein bands are shown on the left and the right of the gel, respectively.

proteins, although a major 140 kDa protein remained (Fig. 1, lane 3). Although the 36 kDa and the 27 kDa proteins were suspected to be derived from the 81 kDa protein reported by Parr *et al.* (1987), we were surprised that no 81 kDa protein band was observed in the heated protein preparation released from the crude peptidoglycan (Fig. 1, lane 3). To positively identify the origin of the 36 kDa and the 27 kDa proteins, we fractionated the peptidoglycan-associated proteins by gel filtration chromatography. SDS-PAGE of the pooled fractions showed an 81 kDa protein (Fig. 1, lane 4) which dissociated into a major 36 kDa protein and minor 27 kDa and 72 kDa proteins after heating at 100 °C (Fig. 1, lane 5). Isolation of the single 81 kDa protein was accompanied by the disappearance of the 140 kDa protein, suggesting that the 81 kDa protein was derived from the 140 kDa protein during the gel filtration chromatography.

High-performance cation exchange chromatography of the gel filtration product supplied a preparation of more than 98% pure 81 kDa protein, as determined by densitometry of Coomassie brilliant blue-stained gels (Fig. 1, lane 6). Dissociation of this 81 kDa protein by heating to 100 °C generated bands of molecular mass 72 kDa, 36 kDa and 27 kDa, although most of the 81 kDa protein remained undissociated (Fig. 1, lane 7). The lack of the 72 kDa band prior to heating (Fig. 1, lane 6) suggested that this band was derived from dissociation of the 81 kDa protein. Dissociation of our purified 81 kDa protein into 36 kDa and 27 kDa species demonstrated that this 81 kDa protein is the major porin oligomer of *B. cepacia* identified by Parr *et al.* (1987) and confirmed by Aronoff (1988) and Burns & Clark (1992). No further dissociation of the oligomer into the two proteins was achieved by prolonged heating (data not shown). These results suggest that this 81 kDa oligomeric protein exists as a tight complex. Burns & Clark (1992) previously designated the 36 kDa protein as OpcP. To represent the relationship between the 81 kDa porin oligomer and its component proteins, we designated the 81 kDa *B. cepacia* porin oligomer, the 36 kDa protein and the 27 kDa protein as OpcPO, OpcP1 and OpcP2, respectively.

Guanidine hydrochloride or urea caused OpcPO to dissociate into OpcP1 and OpcP2 at temperatures lower than 100 °C. Partial dissociation of OpcPO into OpcP1 and OpcP2 at 50 °C in 8 M urea solution (Fig. 2, lane 5) and at 37 °C in 6 M guanidine hydrochloride solution (Fig. 2, lane 7) was observed. Heating to 70 °C in either of these solutions caused the complete disappearance of the band representing OpcPO and enlarged both bands representing OpcP1 and OpcP2 (Fig. 2, lanes 6 and 9), whereas OpcPO was scarcely dissociated even at 70 °C in 1 M NaCl solution (Fig. 2, lane 3). OpcPO was stable following incubation at 37 °C and at 50 °C in guanidine hydrochloride solutions at concentrations less than 5 M and in urea solutions of less than 7 M, respectively (data not shown).

Incubation of purified OpcPO in citrate buffer (pH 3.0) at 50 °C showed an unusual dissociation into two proteins of 72 kDa and 27 kDa (Fig. 3, lane 3). However, no

Fig. 2. Dissociation of OpcPO in the presence of sodium chloride, urea and guanidine hydrochloride. The purified OpcPO (7 µg protein) was diluted with 5 vols 1 M NaCl (lanes 1, 2 and 3), 8 M urea solution (lanes 4, 5 and 6) or 6 M guanidine hydrochloride solution (lanes 7, 8 and 9) and these mixtures were then incubated for 30 min at the following temperatures: lanes 1, 4 and 7, 37 °C; lanes 2, 5 and 8, 50 °C; lanes 3, 6 and 9, 70 °C. After removing NaCl, urea and guanidine hydrochloride by dialysis against pure water, samples were solubilized at 25 °C for 10 min and subjected to SDS-PAGE. Molecular masses of relevant protein bands are shown on the right of the gel.

Fig. 3. Dissociation of OpcPO in citrate buffer. Purified OpcPO (7 µg protein) was diluted with 5 vols 100 mM citrate buffer at the following pH: lanes 1–3, pH 3.0; lanes 4–6, pH 4.0; lanes 7–9, pH 5.0. The mixtures were then incubated overnight at the following temperatures: lanes 1, 4 and 7, 20 °C; lanes 2, 5 and 8, 37 °C; lanes 3, 6 and 9, 50 °C. Finally, samples were solubilized at 25 °C for 10 min and subjected to SDS-PAGE. The arrowhead shows the 27 kDa protein (OpcP2) released from the 81 kDa protein (OpcPO). Molecular masses of relevant protein bands are shown on the right of the gel.

dissociation of OpcPO was observed in the same buffer at pH 4.0 or 5.0, even when incubated at 50 °C (Fig. 3, lanes 4–9). To investigate the cause of this unusual dissociation, OpcPO was incubated at 50 °C in other buffer systems at pH 3.0. In phthalate buffer, OpcPO dissociated completely into OpcP1 and OpcP2 on incubation for 10 h,

Fig. 4. (a) OpcP1 and OpcP2 purified by the Prep Cell system. OpcP1 and OpcP2 derived from the purified OpcPO in 6 M guanidine hydrochloride were fractionated with the Prep Cell system. Lanes: 1 and 2, purified OpcP1; 3 and 4, purified OpcP2. (b) Comparison of mobilities of OpcP1 and OmpF. OmpF was purified from *E. coli* B cells. Lanes: 1 and 2, purified OpcP1; 3 and 4, purified OmpF from *E. coli* B cells. Samples were solubilized at 25 °C for 10 min (lanes 1 and 3) or at 100 °C for 10 min (lanes 2 and 4) and subjected to SDS-PAGE. Molecular masses of relevant protein bands are shown on the right of the gel.

although OpcPO was stable even after incubation for 20 h in both glycine/HCl and acetate buffers (data not shown). Thus, the unusual dissociation of OpcPO into the 72 kDa and 27 kDa proteins is specific for citrate and phthalate buffers at pH 3.0. It is possible that the weak divalent-ion chelating abilities of these two buffers may stimulate the dissociation of OpcPO.

Purification and characterization of OpcP1 and OpcP2

OpcP1 and OpcP2 were independently isolated by the Prep Cell system. The mobility of OpcP2, which eluted earlier than OpcP1 on SDS-PAGE gels, was unaffected by heating (Fig. 4a, lanes 3 and 4). On the other hand, SDS-PAGE of the OpcP1 preparation resulted in the following distinguishable profiles: a band of OpcP1 with the addition of two protein bands of higher molecular mass (72 kDa and 53 kDa) following solubilization at 25 °C (Fig. 4a, b, lanes 1); and a single 36 kDa band following solubilization at 100 °C (Fig. 4a, b, lanes 2). These results indicated that the 72 kDa and the 53 kDa bands observed in the purified OpcP1 preparation were generated by the self-assembly of OpcP1 in buffer containing MEGA-9. Addition of purified OpcP2 into the purified OpcP1 preparation containing the two proteins of higher molecular mass allowed the emergence of the 81 kDa assembly product, presumably representing OpcPO, with accompanying decreases of the 27 kDa, the 36 kDa and the 53 kDa proteins. Moreover, the amount of the 81 kDa product increased with the amount of purified OpcP2 added (Fig. 5).

For comparison, the OmpF trimer, which forms the major diffusion pore in the *E. coli* outer membrane

Fig. 5. Formation of a higher molecular mass product by association of OpcP1 and OpcP2. Purified OpcP1 and OpcP2 prepared in 30 mM sodium phosphate buffer, pH 7.5, containing 2% MEGA-9 were mixed and incubated at 37 °C. After 8 h, the mixtures were solubilized at 25 °C for 10 min and subjected to SDS-PAGE. Lanes: 1, OpcP2 (2.03 µg; 75 pmol as molecular mass 27 kDa); 2, OpcP1 (3.6 µg; 100 pmol as molecular mass 36 kDa); 3, OpcP1 (4.3 µg; 120 pmol) plus OpcP2 (1.62 µg; 60 pmol); 4, OpcP1 (4.3 µg; 120 pmol) plus OpcP2 (3.24 µg; 120 pmol); 5, OpcP1 (4.3 µg; 120 pmol) plus OpcP2 (3.89 µg; 144 pmol). Molecular masses of relevant protein bands are shown on the right of the gel.

(Steven *et al.*, 1977; Nakae *et al.*, 1979; Kameyama *et al.*, 1982; Maezawa *et al.*, 1983), was purified from *E. coli* B and its mobilities tested on the same SDS-PAGE gels. The purified OmpF trimer migrated at the positions corresponding to 72 kDa and 36 kDa after solubilization at 25 °C and 100 °C, respectively (Fig. 4b, lanes 3 and 4).

Immunological characterization of OpcPO, OpcP1 and OpcP2

Immunization of mice with either OpcP1 or OpcP2 purified by the Prep Cell system resulted in a strong antibody response, indicating that these proteins are immunogenic *in vivo*. The dissociated or assembled proteins observed in the above experiments were then characterized by immunoblot assays with each of the polyclonal antisera from the immunized mice.

Antisera from OpcP1- and OpcP2-immunized mice reacted with OpcP1 and OpcPO, and OpcP2 and OpcPO, respectively, in the purified OpcPO preparation (Fig. 6, lanes 3 and 4). Reactions of both these antisera with the heated preparation of outer membrane proteins released from briefly sonicated *B. cepacia* cells exhibited a signal corresponding to a 140 kDa protein as well as the respective OpcP2 and OpcP1 protein, but not to OpcPO (Fig. 6, lanes 1 and 2). No cross-reaction of either sera between OpcP1 and OpcP2 was observed in either of these assays (Fig. 6, lanes 1–4).

Further immunoblotting revealed the following. (i) Anti-

Fig. 6. Identification of dissociated proteins by immunoblot assays. The immunoreactivities of antisera raised against OpcP1 (lanes 1, 3, 5 and 7) and to OpcP2 (lanes 2, 4, 6, and 8) were tested with outer membrane proteins released from briefly sonicated *B. cepacia* cells (lanes 1 and 2), with the purified OpcPO (lanes 3–6), the purified OpcP1 (lane 7) and the purified OpcP2 (lane 8). The antigens were solubilized thus: lanes 1–4, at 100 °C for 10 min; lanes 5 and 6, at 25 °C for 10 min after overnight incubation at 50 °C in 100 mM citrate buffer, pH 3.0; lanes 7 and 8, at 25 °C for 10 min. After fractionation by SDS-PAGE, proteins in the gel were analysed by immunoblot assays with murine polyclonal antisera. The molecular masses of signals are shown by symbols on the right of each lane. ●, 140 kDa; ○, 81 kDa; ▲, 72 kDa; △, 53 kDa; ■, 36 kDa; □, 27 kDa.

serum raised against OpcP1 recognized the 72 kDa protein, but not the 27 kDa protein that is produced from OpcPO after incubation in citrate buffer at pH 3.0 (Fig. 6, lane 5). Antiserum against OpcP2 detected only the 27 kDa protein, but not the 72 kDa protein (Fig. 6, lane 6). (ii) Antiserum specific for OpcP1 reacted with the 72 kDa and the 53 kDa proteins in the preparation of purified OpcP1 (Fig. 6, lane 7), but these proteins were not detected by antiserum raised against OpcP2 (Fig. 6, lane 8). These findings supported the results of the previous dissociation experiment and suggested that OpcP1 has the ability to self-assemble, producing the 72 kDa and the 53 kDa products, and that OpcPO is ultimately produced by the association of a 27 kDa protein, probably OpcP2, with the 72 kDa assembly product.

DISCUSSION

Porin oligomers from Gram-negative bacteria such as *E. coli* and *Salmonella typhimurium* dissociate into their respective homologous protein sub-units (Benz, 1988). However, the 81 kDa protein which we have designated OpcPO in the *B. cepacia* outer membrane dissociates into two proteins with different mobilities on SDS-PAGE gels (Parr *et al.*, 1987). Although this unique heterogeneity of OpcPO has been confirmed by several investigators (Aronoff, 1988; Burns & Clark, 1992), there is little information on the relationship between these proteins. Prior to investigation of the individual functions of the proteins derived from OpcPO, it is important to resolve

the following question to determine the basic relationship between these molecules: are OpcP1 and OpcP2 proteolytic fragments of OpcPO accompanying its dissociation, or does OpcPO contain native heterogeneous subunit proteins?

To resolve the above question, we independently purified OpcP1 and OpcP2 from a preparation of OpcPO. Formation of an assembled product, with a molecular mass corresponding to that of OpcPO from a mixture of purified OpcP1 and OpcP2 (Fig. 5) indicated that OpcP1 and OpcP2 are not proteolytic fragments. The purified proteins were used for the production of murine antisera directed against each to resolve another question: if OpcP2 is a proteolytic product derived from OpcP1, the antiserum directed against OpcP2 should also recognize OpcP1 because OpcP1 should contain OpcP2. Immunoblot assays with both murine antisera showed that no cross-reaction between OpcP1 and OpcP2 (Fig. 6) occurred. Furthermore, the heat-dissociation of OpcP1 into its components generated neither a band corresponding to OpcP2 on SDS-PAGE (Fig. 4) nor a signal corresponding to OpcP2 in the immunoblots (Fig. 6). Failure to detect cross-reactivity between antisera to either OpcP1 or OpcP2, and the lack of demonstrable OpcP2 in the heat-treated OpcP1 preparation indicated that OpcP2 is not a proteolytic derivative of OpcP1, and further that OpcP1 and OpcP2 are immunologically distinguishable component subunits of OpcPO.

Further characteristics of OpcPO and its subunit proteins were determined as follows. Immunoblot assays (Fig. 6) with antisera directed against either OpcP1 or OpcP2 indicated that incubation of OpcPO in citrate buffer at pH 3.0 brought about its dissociation into a 72 kDa oligomer of OpcP1, and a 27 kDa protein, identified as OpcP2. Formation of the 72 kDa product from purified OpcP1 and subsequent heat-dissociation of this 72 kDa product into OpcP1 in the absence of other polypeptides (Fig. 4) supported the conclusion that the 72 kDa protein recovered from citrate buffer at pH 3.0 was an oligomer assembled from OpcP1 alone. The oligomer-forming activity of OpcP1 suggests that OpcPO is itself constructed by a non-covalent association between OpcP2 and the 72 kDa oligomer. The findings presented here indicate that OpcPO is a unique hetero-oligomer composed of two distinguishable subunit proteins in contrast to enteric bacterial porin oligomers, such as OmpF (Steven *et al.*, 1977; Nakae *et al.*, 1979; Kameyama *et al.*, 1982; Maezawa *et al.*, 1983), OmpC (Palva, 1979) and PhoE (Palva & Randall, 1978; Yu *et al.*, 1979), which exist as trimers formed from homologous protein subunits.

The existence of two assembly products of molecular mass greater than that of OpcP1 in the purified OpcP1 preparation suggested that dimers and trimers of OpcP1 may form. If this is true, the 72 kDa product would be expected to migrate at a different position corresponding to a molecular mass of 108 kDa, which can be calculated for a trimer of the 36 kDa protein, on SDS-PAGE. Similarly a dimer of OpcP1 might be expected to migrate to a position equal to 72 kDa. However, there was

substantial disagreement between the mobilities seen by SDS-PAGE and the calculated values. To resolve this problem we examined OmpF from *E. coli*, which exists in a trimeric form with a molecular mass of 108 kDa under normal conditions. Using our separation conditions, the OmpF trimer migrated to a position identical to that of the OpcP1 72 kDa oligomer on the same gel (Fig. 4b). This suggested that the true molecular mass of the trimer of OpcP1 may be similar to that of OmpF, i.e. 108 kDa.

SDS-PAGE of the heat-treated OpcPO preparation (Fig. 1) indicated that the content of OpcP2 in the OpcPO molecule is less than that of OpcP1. Our results suggest that OpcPO is formed from three molecules of OpcP1 and one or two molecule(s) of OpcP2. Detection of a 140 kDa protein in the immunoblots of the preparation of outer membrane proteins released from the *B. cepacia* cells by brief sonication (Fig. 6) indicates that OpcPO is present as a higher molecular mass aggregate in the native outer membrane. This is supported by the observation that the 140 kDa protein, but not the 81 kDa species, occurred in the protein preparation released from crude peptidoglycan, and that this 140 kDa protein could be dissociated into OpcP1 and OpcP2 by heating.

In summary, we have demonstrated that OpcPO, a major porin oligomer from *B. cepacia*, is formed by the non-covalent association of two distinct subunit proteins, OpcP1 and OpcP2.

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