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Porins of *Vibrio cholerae*: Purification and Characterization of OmpU

SUBHRA R. CHAKRABARTI,[1] KEYA CHAUDHURI,[1]* KEYA SEN,[2] AND JYOTIRMOY DAS[1]

Biophysics Division, Indian Institute of Chemical Biology, Calcutta, India,[1] and Department of Medicine & Microbiology, State University of New York at Buffalo, Buffalo, New York 14215[2]

Received 1 August 1995/Accepted 27 October 1995

Three outer membrane proteins with molecular masses of 40, 38, and 27 kDa of the hypertoxinogenic strain 569B of *Vibrio cholerae* have been purified to homogeneity. The synthesis of all the three proteins is regulated by the osmolarity of the growth medium. The pore-forming ability of the 40-kDa protein, OmpT, and the 38-kDa protein, OmpU, has been demonstrated by using liposomes, in which these proteins were embedded. The 27-kDa protein, OmpX, though osmoregulated, is not a porin. OmpU constitutes 30% of the total outer membrane protein when grown in the presence of 1.0% NaCl in the growth medium and 60% in the absence of NaCl. OmpU is an acidic protein and is a homotrimer of 38-kDa monomeric units. Its secondary structure contains predominantly a β-sheet, and three to four Ca^{2+} ions are associated with each monomeric unit. Removal of Ca^{2+} irreversibly disrupts the structure and pore-forming ability of the protein. The pore size of OmpU is 1.6 nm, and the specific activity of the OmpU channel is two- to threefold higher than that of *Escherichia coli* porin OmpF, synthesis of which resembles that of OmpU with respect to the osmolarity of the growth medium. The pore size of OmpT, which is analogous to OmpC of *E. coli*, is smaller than that of OmpU. Southern blot hybridization of *V. cholerae* genomic DNA digested with several restriction endonucleases with nick-translated *E. coli ompF* as the probe revealed no nucleotide sequence homology between the *omp*U and *ompF* genes. OmpU is also not antigenically related to OmpF. Anti-OmpF antiserum, however, cross-reacted with the 45-kDa *V. cholerae* outer membrane protein, OmpS, the synthesis of which is regulated by the presence of maltose in the growth medium. OmpU hemagglutinated with rabbit and human blood. This *tox*R-regulated protein is one of the possible virulence determinants in *V. cholerae* (V. L. Miller and J. J. Mekalanos, J. Bacteriol. 170:2575–2583, 1988).

The outer membrane of enteric pathogens is one of several factors that are involved in the interaction between the bacterium and the epithelial cell surface and confers resistance to the bacterium to bile salts and to host defense factors such as lysozyme and leukocyte proteins (20). To understand the regulatory network involved in the host-parasite interaction and its possible role in virulence, the outer membrane of *Vibrio cholerae*, a noninvasive gram-negative bacterium and the etiological agent of cholera, has been examined by several investigators (9, 10, 13, 14). Some atypical features of the cell surface of *V. cholerae* have emerged from these studies. Normally, gram-negative bacteria with smooth-type lipopolysaccharide (LPS) in the outer membrane are resistant to hydrophobic compounds and detergents (34). Although *V. cholerae* cells possess smooth LPS (3), these cells are highly sensitive to a wide range of chemicals, particularly hydrophobic compounds and neutral and anionic detergents. This was attributed partly to the presence of phospholipids in the outer leaflet of the outer membrane, at least in hypertoxinogenic strain 569B of *V. cholerae* (23), thereby allowing the entry of nonpolar molecules by diffusion. The LPS of *V. cholerae* contains O-antigenic sugars and has less negative charge than those in other organisms (23). The murein network of *V. cholerae* is weak, and the cells lyse rapidly in hypotonic medium and in the presence of chelating agents (10). The outer membrane can be isolated directly from whole cells by treatment with protein denaturants such as urea at room temperature (9). Unlike other gram-negative organisms, *V. cholerae* cells are equally sensitive to penicillin and ampicillin and in general are more sensitive than *Escherichia coli* to most of the beta-lactam antibiotics, resulting in adaptive mutation and beta-lactam-resistant cells' emerging at a high frequency (31). A beta-lactam-induced 25-kDa protein which might be responsible for conferring resistance to beta-lactam antibiotics by interfering with OmpU, through which the beta-lactam antibiotics normally enter the cell, has recently been identified in the outer membrane of beta-lactam-resistant cells (5).

Five to six major outer membrane proteins have been reported in *V. cholerae*, of which the 45-kDa protein OmpS (8) and OmpV (33), a 23- to 25-kDa protein, have been examined in detail. OmpS is a maltoporin, the synthesis of which is enhanced at least fivefold in medium containing maltose. The promoter region of *ompS* is homologous to the *malK-lamB* promoter, and the expression of *ompS* is controlled by MalT in *E. coli* (8). OmpV is a highly immunogenic protein (14, 28) and is the only heat shock protein of *V. cholerae* that is located in the outer membrane (28). The 35-kDa outer membrane protein is heat modifiable and represents the OmpA-like protein of *E. coli* (1). The functions of other major outer membrane proteins, namely, OmpT, OmpU, and OmpX, are not known. From indirect evidence, such as osmoregulation and trypsin insensitivity, it has been suggested that OmpT and OmpU might be porins that form water-filled channels across the membrane (10, 14).

The *toxR* gene product of *V. cholerae*, a transmembrane DNA-binding protein, activates both directly and indirectly the expression of multiple virulence properties (6, 15). OmpT and OmpU are in the ToxR regulon. Mutations in the *toxR* gene alter the outer membrane protein profile and the process of osmoregulation of OmpT and OmpU (15). Because of their possible role in pathogenesis, characterization of these two proteins was considered important. Strain 569B is considered

* Corresponding author. Mailing address: Biophysics Division, Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Rd., Calcutta 700 032, India. Phone: (091-33) 473 0350. Fax: (091-33) 473 0350 or 473 0284. Electronic mail: iicb%sirnetc@sirnetd.ernet.in.

unusual from the point of view of regulation of virulence determinants because this strain is independent of ToxR and ToxS for the expression of the ToxR regulon, unlike other toxinogenic *V. cholerae* strains. It is in this context that the present report describes the purification of OmpT and OmpU from strain 569B and a detailed characterization of OmpU.

MATERIALS AND METHODS

Chemicals and enzymes. Dansyl chloride, Tris, all electrophoretic reagents, standard proteins, DNase, RNase, dithio-bis-succinimidyl propionate, phosphatidylcholine, and diacetyl phosphate were purchased from Sigma. Sarkosyl NL97 was purchased from Geigy Industrial Chemicals. Radiochemicals used were either from Bhabha Atomic Research Centre, Trombay, India, or from Amersham Inc. High-pressure liquid chromatography (HPLC) buffers were degassed and purged with nitrogen to prevent oxidation of proteins, particularly at high or low pH.

Bacterial strains and growth conditions. Hypertoxinogenic strain 569B of *V. cholerae* (biotype, classical; serotype, Inaba), which carries a streptomycin resistance marker (50 μg/ml), was used in this study. Cells were grown at 37°C in nutrient broth, pH 8.0, with shaking and maintained as described previously (27). Whenever necessary, the growth medium was supplemented with 2% NaCl or 2% maltose. The *E. coli* strains were grown in Luria broth at pH 7.4. Cell viability was assayed as CFU on nutrient agar plates, and growth was monitored by measuring the A_{585}; 8.0×10^8 CFU/ml corresponded to an A_{585} of 1.0.

Preparation of crude cell envelope and outer membrane. *V. cholerae* cells at different stages of growth were harvested by centrifugation (6,000 × *g*, 5 min) and suspended in 20 mM Tris-HCl buffer (pH 7.6) containing 5 mM phenylmethylsulfonyl fluoride. Cells were disrupted in an ultrasonic disintegrator by 30-s pulses at 55 W until the cell suspension became translucent. The cell lysate was treated with DNase and RNase (100 μg/ml) for 20 min at 37°C, and the unbroken cells were removed by centrifugation. The cell lysate was centrifuged in a Beckman Ti-50 rotor for 30 min at 105,000 × *g*. The supernatant was discarded, and the pellet containing the crude cell envelope was washed with cold phosphate buffer, suspended in the same buffer, and kept at −20°C. The outer membrane along with the associated peptidoglycan (PG) layer was isolated from the crude cell envelope by treatment with 1% (wt/vol) Sarkosyl NL-97 for 30 min at 25°C, followed by centrifugation at 105,000 × *g* for 1 h. Inner membrane contamination of the outer membrane preparation was estimated by measuring the cytochrome *b* content, which is located in the inner membrane and was therefore used as a marker. All the outer membrane preparations used had less than 5% inner membrane contamination.

Preparation of PG-associated proteins. To identify proteins that are associated with the PG, the outer membrane preparation was solubilized by the method of Rosenbusch (26). Purified outer membrane in 10 mM Tris-HCl (pH 7.6) buffer containing 2% sodium dodecyl sulfate (SDS) was incubated at 30°C for 30 min and then centrifuged at 105,000 × *g* for 1 h at 15°C. The pellet was suspended in 0.4 M NaCl–5 mM EDTA–3 mM sodium azide–0.05% β-mercaptoethanol and incubated at 37°C for 2 h. Following incubation, the samples were centrifuged at 105,000 × *g* for 1 h at 15°C, and the supernatant containing the PG-associated proteins was kept at 100°C for 2 min and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Protein purification. Purified outer membrane was extracted with 1% Sarkosyl NL-97, and the pellet was washed several times in 20 mM Tris-HCl (pH 7.6). The washed pellet was suspended in the same buffer containing 4% Triton X-100, incubated at 37°C for 30 min, and centrifuged at 105,000 × *g* for 60 min at 4°C. The supernatant was passed through a DEAE-cellulose column (12 by 2 cm) equilibrated with 20 mM Tris-HCl (pH 7.6) buffer containing 0.1% Triton X-100. The proteins were eluted from the column by a continuous gradient of 0 to 0.1 M NaCl in 20 mM Tris-HCl (pH 7.6) containing 0.1 M Triton X-100, and 1-ml fractions were collected. Each fraction was examined by SDS-PAGE, and fractions containing a particular protein were pooled and concentrated with a Centricon-3 (Amicon Inc.) concentrator. The 45-kDa (OmpS), 40-kDa (OmpT), and 27-kDa (OmpX) proteins were found to be pure after this step. The 38-kDa (OmpU) protein had minor contamination with the 27-kDa protein. To remove the contaminating 27-kDa protein, the concentrated fraction containing OmpU was loaded on a fast protein liquid chromatography (FPLC) (Superose-12) column equilibrated with the same buffer. The protein was eluted from the column with the same buffer, and fractions were collected. Each fraction was assayed, and the protein content was estimated with Micro-BCA reagent (Pierce), with bovine serum albumin as the standard. *E. coli* OmpF was purified from strain CM6 by the method of Nikaido (17).

Determination of molecular weight and Stokes radius of OmpU. To determine the molecular weight of the protein, OmpU in 20 mM Tris-HCl (pH 7.6) was passed through a TSKG 3000 SW HPLC column (0.75 by 30 cm; Pharmacia). The column was equilibrated with 20 mM Tris-HCl (pH 7.6) and precalibrated with standard gel filtration markers.

Electrophoresis. SDS-PAGE was carried out by the method of Laemmli (7). For a better resolution of OmpT and OmpU, the method of Lugtenberg et al. (11) was used. Electrophoresis was carried out at a constant current (15 mA/cm), and the gel was stained with 0.25% Coomassie blue.

Isoelectric focusing. Isoelectric focusing was carried out in nondenaturing slab gels of 12.5% polyacryalmide containing ampholines (pH 3.0 to 10.0; Pharmacia) (22). The pH gradient was calibrated by using isoelectric point markers (Pharmacia). The gels were fixed in 12% trichloroacetic acid–30% methanol–3.5% salphosalicylic acid and stained with silver (29).

Amino acid analysis and sequencing. Protein (20 μmol) was hydrolyzed with 6 N HCl at 110°C for 24 h in a sealed glass tube flushed with nitrogen with traces of phenol. The amount of tryptophan was measured by using *N*-bromosuccinimide (NBS) (2). In this method, purified protein in 70% acetic acid at a concentration of 1 mg/ml was taken in a cuvette. NBS in aqueous solution (10^{-2} M) was then added (5.0 μl) to the cuvette with stirring. After several minutes, the spectrum of the protein solution is recorded to measure the absorption at 280 nm. No change in optical density (OD) was observed. The amount of tryptophan in lysozyme was measured by the same method.

To sequence the N-terminal end of OmpU, to 2 mg of the protein in 0.2 M $NaHCO_3$ (pH 9.0), 0.1 ml of dansyl chloride (10 mg/ml) in acetone was added. The reaction mixture was incubated in the dark at 37°C for 16 h, dialyzed exhaustively, and dried in vacuum. The dansylchloride-protein complex was hydrolyzed with 1 ml of 5.7 N HCl at 105°C for 22 h. After removal of HCl, the hydrolysate was taken in a few drops of 50% pyridine and subjected to thin-layer chromatography. The standard dansylated amino acids used as markers were spotted on the same plate. The chromatography was performed with either benzene-pyridine-acetic acid (16:4:1) or *n*–butanol–pyridine–acetic acid-water (30:20:6:24) solvent in the dark. The plates were dried, and fluorescence was viewed under UV light. Sequencing was done in an automated amino acid sequencer (Applied Biosystems model 473A).

Cross-linking. Purified proteins in 0.2 M triethanolamine, pH 8.5, buffer were treated with different concentrations of dithio-bis-succinimidyl propionate in dimethyl sulfoxide at 22°C. After 30 s, the reaction was stopped by adding an excess amount of 1 M Tris-HCl, pH 8.5, buffer. Samples were then extensively dialyzed against buffer containing 20 mM Tris-HCl (pH 7.6) and 0.1% Triton X-100 to remove triethanolamine.

Spectroscopic methods. For circular dichroism (CD) spectroscopy, the purified protein in 20 mM Tris-HCl, pH 7.6 (with or without 10 mM EGTA [ethylene glycol tetraacetic acid]), was taken in quartz cuvettes with a 1-cm path length. Molar ellipticity was recorded between 200 and 250 nm with a JASCO 700 Spectropolarimeter at 25°C. The estimation of alpha-helix and beta-sheet contents was done as described by Chen and Yang (4).

Detection of Ca^{2+}. The presence of Ca^{2+} ions in OmpU was detected by atomic absorption spectrometry of protein samples in 20 mM Tris-HCl, pH 7.6, containing 0.1% Triton X-100.

Liposome swelling assay. The liposome swelling assay was carried out by the method of Nikaido et al. (21). To prepare liposomes or proteoliposomes, 2 ml of benzene was added to 2.4 μmol of acetone-washed egg phosphotidylcholine and 0.2 μmol of diacetyl phosphate and dried under a stream of nitrogen. To the dried sample, 2 ml of diethyl ether was added and dried in a nitrogen atmosphere. The dried sample was suspended either in water or in the solution of desired protein in water. The sample was dried and finally suspended in 0.6 ml of 17% (wt/vol) dextran T-40 (Pharmacia) in 5 mM Tris-HCl, pH 7.5. Twenty microliters of proteoliposomes was mixed rapidly with 0.6 ml of isoosmotic solutions of different sugars, such as arabinose, rhamnose, sucrose, raffinose, and stachyose, in 5 mM Tris-HCl (pH 8.0), and the absorbance at 400 nm was recorded. The isoosmotic concentration of each sugar solution was determined by diluting liposomes with various concentrations of the sugar solution. The concentration which caused neither swelling nor shrinking of the liposomes was taken as the isoosmotic concentration.

Antisera against OmpU and OmpF. Antisera against OmpU and OmpF were raised in rabbits by injecting subcutaneously 50 μg of pure protein in complete Freund's adjuvant at monthly intervals for 3 months. The serum was used at a dilution of 1:200 unless otherwise mentioned.

Nick translation. About 1 μg of a 1.73-kb *Pst*I fragment of the *ompF* gene in plasmid pKS4 (30) was nick translated by using [α-^{32}P]dCTP and DNA polymerase I. The reaction was carried out at 16°C for 1 h, and the nick-translated DNA was separated from the unincorporated [α-^{32}P]dCTP by passage through a Sephadex G-50 column.

Hybridization. Dot blot and Southern blot analyses were done by the methods of Maniatis et al. (12). For Western blot (immunoblot) hybridization, outer membrane proteins of *V. cholerae* 569B and *E. coli* K-12 were separated by SDS-PAGE and transferred to nitrocellulose in a Transblot apparatus (Bio-Rad) as described by Towbin et al. (35). The blots were incubated for 2 h with either rabbit anti-OmpF serum or rabbit anti-OmpU serum followed by alkaline phosphatase-conjugated anti-rabbit immunoglobulin G. Antigens were detected with the Proto Blot system (Promega).

Hemagglutination assay. Sheep, rabbit, and human blood cells were stored at 4°C for not more than 1 week before use. When required for assay, blood cells were washed three times in 0.9% saline, and a 2% suspension was prepared. All hemagglutination studies were performed in microtiter plates by adding 50 μl of OmpU (500 μg/ml) in 20 mM Tris-HCl (pH 7.6) and making twofold serial dilutions in 0.9% NaCl. A 50-μl portion of erythrocytes was added to each well, and the plates were incubated at 4°C overnight. The highest dilution giving

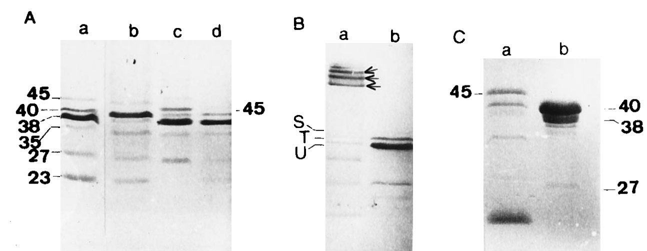

FIG. 1. SDS-PAGE of the outer membrane proteins of *V. cholerae* 569B. (A) Protein profile of the outer membrane extracted from cells grown in nutrient broth containing no NaCl (lane a) or 0.4 M NaCl (lane b) and with maltose (lane c) and without maltose (lane d). Numbers in the margin indicate molecular mass (in kilodaltons). (B) The outer membrane was solubilized in SDS-PAGE sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 1% β-mercaptoethanol, 1% SDS) without heating (lane a) or after heating at 100°C for 2 min (lane b). The arrows indicate the positions of the oligomeric proteins. S, T, and U indicate the positions of monomeric OmpS, OmpT, and OmpU, respectively. (C) PG-associated proteins were isolated as described in the text and analyzed by SDS-PAGE. Lanes: a, outer membrane proteins not associated with PG; b, PG-associated proteins. Numbers in the margin indicate molecular mass (in kilodaltons).

complete hemagglutination was recorded as the endpoint. For the hemagglutination inhibition assay, purified OmpU was incubated with a 1% (wt/vol) solution of different sugars in 0.9% NaCl. Hemagglutination was assayed by performing the test as described above. Inhibition was defined as ≥50% reduction in agglutination compared with the test without the sugar.

RESULTS

Outer membrane proteins of *V. cholerae*. As reported previously (5, 10, 31), six major outer membrane proteins, OmpS (45 kDa), OmpT (40 kDa), OmpU (38 kDa), OmpX (27 kDa), OmpV (23 kDa), and an OmpA-like protein (35 kDa), were resolved when purified outer membrane of strain 569B was analyzed by SDS-PAGE (Fig. 1A, lane a). OmpT, OmpU, and OmpX are osmoregulated, OmpT being synthesized in larger amounts at high osmolarity (0.4 M NaCl) (Fig. 1, lane b). OmpU and OmpX are synthesized more at a low osmolarity of the growth medium (Fig. 1, lane a). The 25-kDa protein reported by Manning and Haynes (14) is presumably the same as the 23-kDa protein reported here, and the difference in size might be due to experimental variation. The synthesis of OmpS in strain 569B increased by more than fivefold in the presence of maltose in the growth medium (Fig. 1A, lane c), as reported for strain X28214 (8).

When the purified outer membrane of *V. cholerae* was analyzed by SDS-PAGE without prior boiling in solubilization buffer, OmpS, OmpT, and OmpU could not be detected in the gel at 45, 40, and 38 kDa, respectively. Instead, some high-molecular-weight proteins were resolved in the gel (Fig. 1B, lane a, arrows). These high-molecular-weight proteins represent the multimeric forms of OmpS, OmpT, and OmpU and are dissociated following boiling of the membrane preparations (Fig. 1B, lane b). This was confirmed by two-dimensional SDS-PAGE of heated and nonheated outer membrane preparations (data not shown).

To examine whether any of the *V. cholerae* outer membrane proteins is associated with PG, purified outer membrane in 10 mM Tris-HCl (pH 7.6)–2% SDS was incubated at 37°C for 30 min, and PG-associated proteins were isolated as described in Materials and Methods and analyzed by SDS-PAGE (Fig. 1C, lane b). OmpT, OmpU, and OmpX of *V. cholerae* are noncovalently associated with the PG layer. Unlike *E. coli* LamB, the OmpS of *V. cholerae* is not associated with PG (Fig. 1C, lane a). PG association, resistance to trypsin digestion (1), and osmoregulation of OmpS, OmpU, OmpT, and OmpX suggest that these proteins represent the porins of *V. cholerae*. OmpS has recently been shown to be a porin (8). In the case of OmpT and OmpU, only indirect evidence suggests that they might be porins, and their pore-forming abilities have not been examined yet.

Purification of OmpT, OmpU, and OmpX. Purified outer membrane of *V. cholerae* was solubilized with 4% Triton X-100 instead of SDS, which is normally used for the purification of porins (17), because OmpU denatured irreversibly when extracted with SDS. The Triton X-100-soluble fraction of the outer membrane (Fig. 2, lane a) was passed through a DEAE-cellulose column, and proteins were eluted with a linear 0 to 0.1 M NaCl gradient in 20 mM Tris-Cl (pH 7.6)–0.1% Triton X-100. The chromatogram revealed four peaks, which were analyzed separately by SDS-PAGE. The peaks eluting at 0.05, 0.07, 0.09, and 0.1 M NaCl corresponded to proteins of 27 (OmpX), 38 (OmpU), 40 (OmpT), and 45 (OmpS) kDa (Fig. 2, lanes c, d, and e). OmpS, OmpT, and OmpU were recovered as oligomers from the column and were more than 95% pure, as determined by densitometric scanning of gels stained with Coomassie blue. OmpU had minor contamination with the 23- and 27-kDa proteins. These impurities were removed by passing the fractions containing OmpU through a Superose-12 FPLC column. The proteins were stable for months when kept in 20 mM Tris-HCl (pH 7.6)–0.1% Triton X-100 at 4°C.

OmpT and OmpU are porins. To investigate whether OmpT, OmpU, and OmpX are porins of *V. cholerae*, purified proteins were reconstituted into liposomes, and the diffusion of arabinose and stachyose through the proteoliposome was monitored spectrophotometrically from the swelling of proteoliposomes as described in Materials and Methods. In some experiments, fragments of outer membrane were used for the constitution of the proteoliposome. The rate of diffusion of arabinose and stachyose through proteoliposomes containing *V. cholerae* outer membrane and several outer membrane proteins reveals that the OmpU trimer contributes significantly to the permeability of the *V. cholerae* outer membrane (Table 1). Both arabinose and stachyose diffused through the proteoliposomes containing OmpU trimer. OmpT trimer, on the other hand, was impermeable to stachyose. When monomeric forms of OmpU, OmpT, or the 27-kDa proteins were used to construct the proteoliposome, no swelling of the liposome occurred even when the permeation of the smallest sugar, arabinose, was examined (Table 1). Thus, only the oligomeric forms of OmpT and OmpU have pore-forming ability. The 27-kDa protein (OmpX), though osmoregulated and trypsin resistant, is not a porin. The rate of diffusion of arabinose through liposomes containing OmpU was much higher than that of liposomes containing equal moles of OmpT (Table 1). To rule out the possibility that the observed difference in the rate of

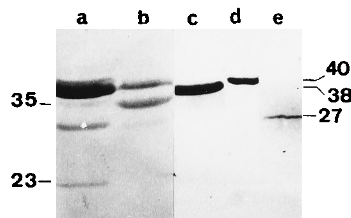

FIG. 2. SDS-PAGE of outer membrane proteins at different stages of purification. Fractions: 4% Triton X-100 solubilized (lane a) and insoluble (lane b). OmpU (lane c), OmpT (lane d), and OmpX (lane e) were tested after DEAE-cellulose chromatography of Triton X-100-soluble outer membrane proteins. Sizes are shown in kilodaltons.

TABLE 1. Relative diffusion rates of arabinose and stachyose through proteoliposomes containing *V. cholerae* outer membrane and various outer membrane proteins[a]

Proteoliposome	Relative rate of diffusion (% of control)	
	Arabinose	Stachyose
OmpU trimer (control)	100	5.8
OmpT trimer	61	0
OmpF trimer	40	0
OmpU monomer	0	0
OmpT monomer	0	0
27-kDa protein (OmpX)	0	0

[a] The rates of diffusion of arabinose and stachyose were 0.11 and 0.07 OD unit/min, respectively, when *V. cholerae* outer membrane (25 μg) were used to construct the proteoliposomes. The rate of diffusion of arabinose (0.24 OD unit/min) when OmpU trimers (20 μg) were used to construct the proteoliposome was taken as 100%. Monomers were prepared by heating trimers for 5 min at 75°C.

permeation between OmpU and OmpT is an artifact arising during the construction of proteoliposomes, the effect of protein concentration on the diffusion rate of arabinose was determined. The diffusion rate of the sugar increased linearly with the amount of protein incorporated into the liposome. Thus, the difference in the diffusion rate between OmpT and OmpU is due to differences in the pore size and/or pore structure.

To determine the pore size of OmpU, the rates of diffusion of uncharged saccharides of different molecular sizes through liposomes containing OmpU were measured (Fig. 3). Taking the diffusion rate of arabinose as 100, the exclusion limit for *V. cholerae* OmpU was estimated to be about 850 Da. From the relative diffusion rate and hydration radii of sugars (16), the pore size of OmpU was found to be 1.6 nm, while the pore size of OmpF of *E. coli* was reported to be 1.2 nm (18). The specific activity of the OmpU channel for arabinose diffusion (defined as change in OD per minute times 1,000 per milligram of protein [36]) was 2.5 times higher than that of *E. coli* OmpF. *V. cholerae* OmpU is thus characterized by higher specific activity and larger pore size than *E. coli* OmpF.

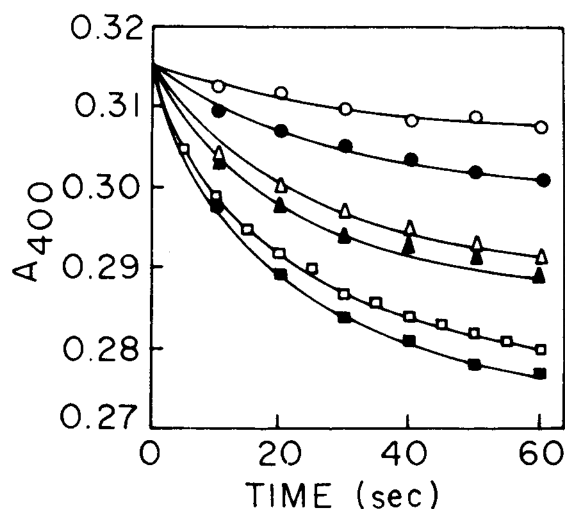

FIG. 3. Liposomes were prepared as described in Materials and Methods, and OmpU was incorporated into the liposome. The ability of different uncharged saccharides to permeate these proteoliposomes was monitored by measuring the change in absorbance at 400 nm over time. ■, water; □, arabinose (150 Da); ▲, rhamnose (165 Da); △, sucrose (342 Da); ●, raffinose (504 Da); ○, stachyose (666 Da).

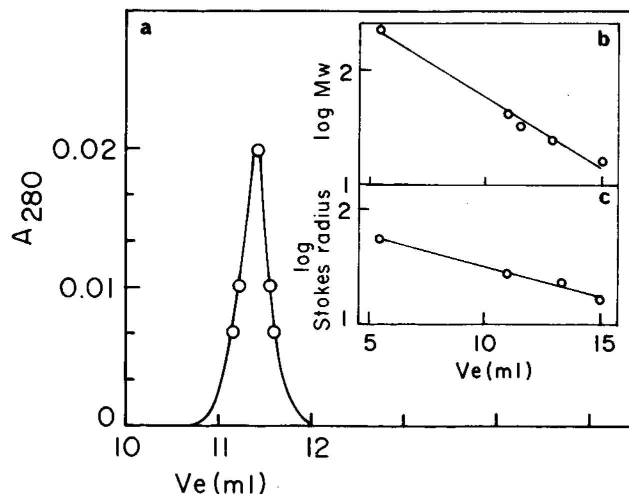

FIG. 4. (a) Elution profile of OmpU from the size exclusion (SE) HPLC column. The protein obtained from a Superose-12 FPLC column (50 μg) was loaded onto a TSKG 3000 SW SE-HPLC column (LKB) and eluted with 20 mM Tris-HCl (pH 7.5) buffer at a flow rate of 0.5 ml/min. The peak (V_e = 11.5 ml) contained OmpU. (b) Dependence of molecular weight (Mw) on elution volume (V_e). The HPLC column was calibrated with catalase (240,000 Da), ovalbumin (45,000 Da), glyceraldehyde-3-phosphate dehydrogenase (36,000 Da), carbonic anhydrase (29,000 Da), and cytochrome *c* (12,500 Da). (c) Dependence of Stokes radius (ϒs) on V_e. Catalase (ϒs = 52 Å [5.2 nm]), ovalbumin (ϒs = 31.2 Å [3.12 nm]), carbonic anhydrase (ϒs = 25.1 Å [2.51 nm]), and cytochrome *c* (ϒs = 17 Å [1.7 nm]) were used as standards.

Characterization of OmpU. The size of the oligomeric form of OmpU was estimated to be 110 kDa by gel filtration. The monomeric unit eluted as a single peak in size exclusion HPLC (Fig. 4a). With a TSKG 3000 SW column, the molecular mass of the monomer was estimated to be 38 kDa (Fig. 4b). The Stokes radius of OmpU monomers was 29 Å (0.29 nm) (Fig. 4c), and the isoelectric point was 3.6. The active form of OmpU thus comprises three identical subunits. OmpU is stable for months when kept in 20 mM Tris-HCl (pH 7.6)–0.1% Triton X-100. In the absence of Triton X-100, the protein precipitates after 3 to 4 h.

Amino acid analysis showed that the number of hydrophilic residues in OmpU is higher than the number of hydrophobic residues (Table 2), and the protein might be rich in acidic amino acids, as revealed by its isoelectric point. The molecular weight of the monomer estimated from the amino acid composition was in agreement with that obtained from SDS-PAGE and gel filtration. Although the number of tyrosyl residues is relatively high, the absorption spectrum of OmpU exhibited a broad peak near 280 nm. This might be due to the absence of tryptophan in the protein. This was confirmed by exciting OmpU at 295 nm and recording the fluorescence emission spectra. The tryptophan-specific emission in the region from 320 to 330 nm could not be detected. Furthermore, there was no change in absorbance at 280 nm when the protein in 70% acetic acid was treated with *N*-bromo-succinimide. Under identical conditions, lysozyme showed a decrease in absorbance at 280 nm corresponding to six tryptophan residues, as reported before (32).

The sequence of the first 20 amino acid residues from the N-terminal end of the protein is DEINQSDAKAVSFVYQA KGE. A 33-mer oligonucleotide sequence deduced from the amino acid sequence by using *V. cholerae* codon bias was syn-

TABLE 2. Amino acid composition

Amino acid	Molar ratio per molecule (integer)
Ala	30
Glx	45
Met	12
Tyr	28
Arg	6
Gly	33
Phe	22
Val	21
His	7
Pho	14
Asx	30
Ile	36
Ser	29
Cys	6
Leu	9
Thr	16
Lys	20
Trp	0
Total	364

thesized, end labeled, and used as a probe in Southern blot hybridization of *V. cholerae* DNA restricted with several restriction enzymes to generate a minibank for identifying the gene encoding OmpU. This work is in progress.

The CD spectra monitored in the far UV region (200 to 250 nm) of the trimeric form of OmpU in 20 mM Tris-HCl (pH 7.6) showed maximum ellipticity at 218 nm (Fig. 5a), characteristic of proteins having a significant amount of beta-sheeted structure. The alpha-helix, beta-sheet, beta-turn, and random-coil contents of OmpU were estimated to be 7.9, 55.5, 16.3, and 19.8%, respectively. In the presence of 10 mM EDTA or EGTA, the beta-sheeted structure was irreversibly disrupted, and the protein assumes a largely random-coil conformation (Fig. 5b). The alpha, beta, and random-coil conformations of EDTA/EGTA-treated protein were 19.1, 0.8, and 80.1%, respectively. The beta-sheeted structure could not be restored upon removal of EDTA/EGTA. In the presence of EDTA/EGTA, the purified protein migrated faster than the monomers in SDS-PAGE (Fig. 5, inset, lane C). This is in contrast to *Rhodopseudomonas sphaeroides* porin, which comigrated with the monomeric units following treatment with EDTA (37). Atomic absorption spectrophotometry of OmpU showed that three to four Ca^{2+} ions are associated with each monomeric unit. Thus, Ca^{2+} ions might be involved in maintaining the beta-sheeted structure of OmpU.

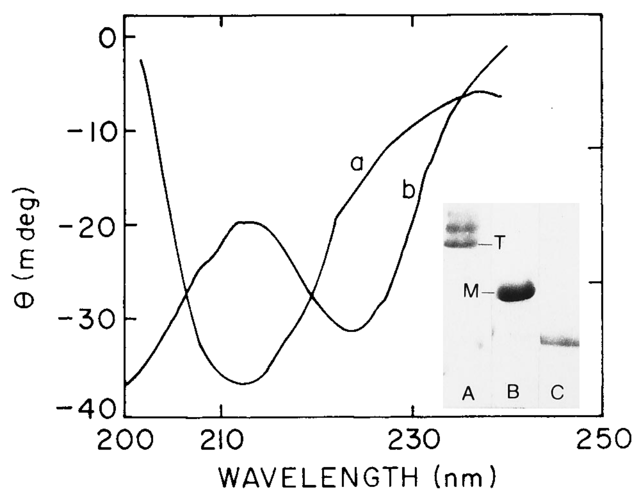

FIG. 5. CD spectra of OmpU. The protein (50 μg/ml) in 20 mM Tris-HCl (pH 7.6) buffer was scanned in the far UV region (200 to 250 nm) at 25°C (curve a). Purified active OmpU was treated with 10 mM EGTA, and the spectrum was taken under identical conditions (curve b). (Inset) SDS-PAGE of trimer and multimer (lane A), monomer (lane B), and trimer treated with 10 mM EGTA (lane C). T and M, trimers and monomers, respectively.

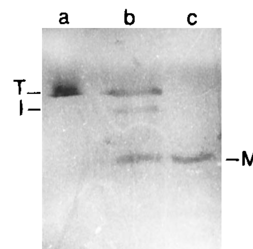

FIG. 6. Transition from trimer to monomer. OmpU in 20 mM Tris-HCl was exposed for 5 min at 40°C (lane a), 50°C (lane b), and 75°C (lane c); cross-linked as described in Materials and Methods; and analyzed by SDS-PAGE. T, I, and M, trimer, intermediate conformation, and monomer, respectively.

Interactions involved in trimer formation. To examine whether the monomers of OmpU are held together by covalent linkages, the trimeric form was analyzed by SDS-PAGE in the presence and absence of β-mercaptoethanol in the sample buffer. In both cases, only the monomeric form of OmpU could be resolved in the gel. This excludes the possibility of -S-S- linkages between the monomers in the trimer. Buffers containing different concentrations of NaCl or KCl (0.5 to 2.0 M) failed to dissociate the trimer, ruling out electrostatic interaction as the major force involved in trimerization. Presumably, hydrophobic interactions are involved in maintaining the trimeric form.

OmpU in 20 mM Tris-HCl (pH 7.6)–0.1% Triton X-100 was exposed for 5 min to temperatures ranging from 30 to 90°C and cross-linked with dithio-bis-succinimide propionate. The samples were boiled in solubilizing buffer without β-mercaptoethanol or dithiothreitol and analyzed by SDS-PAGE. Up to 40°C, the protein maintained its trimeric form (Fig. 6, lane a). At 50°C, an intermediate was resolved in the gel along with trimers and monomers (Fig. 6, lane b). The nature of this intermediate is not known. It is possible that this represents a partially unfolded form of the trimer. Above 75°C, the protein was resolved exclusively as monomers (Fig. 6, lane c).

Hemagglutinating activity of OmpU. The hemagglutinating activity, which is often considered a useful index of the colonization potential of pathogens, of purified OmpU was examined in rabbit, sheep, and human erythrocytes. OmpU showed mannose- and galactose-inhibitable hemagglutination with rabbit and human blood but not with sheep blood. The hemagglutinating activity of OmpU led us to examine the possibility of glycosylic linkages in OmpU by using periodic acid-Schiff (PAS) staining (39). Purified OmpU could be stained with PAS. To confirm the presence of sugar moieties in OmpU, the purified protein was analyzed by gas-liquid chromatography, and the sugars were identified by coinjection with alditol acetate of standard sugars. The chromatogram showed the presence of galactose, mannose, glucose, *N*-acetylglucosamine, and traces of heptose. This is not unexpected in view of the fact that LPS binds tightly to porins and is known to remain bound even with largely denatured monomeric porins during SDS-PAGE

(25). Gas-liquid chromatography of purified LPS of *V. cholerae* 569B showed the presence of all the sugars that are associated with OmpU except galactose.

Lack of nucleotide sequence homology between *ompU* and *ompF*. To examine the nucleotide sequence homology between *ompU* and *ompF*, the nick-translated *E. coli ompF* gene was used as the probe in Southern blot hybridization of *V. cholerae* DNA digested with several restriction enzymes. Even under very low stringency conditions, the *E. coli ompF* probe did not hybridize with *V. cholerae* DNA. Thus, extensive nucleotide sequence divergence exists between the two genes. To investigate whether this is typical of the *ompU* gene of strain 569B or represents a general feature of *V. cholerae ompU*, the *E. coli ompF* probe was used in dot blot hybridization with several *V. cholerae* strains belonging to different serovars and biotypes. The DNA from none of the strains examined hybridized with *ompF*. Restriction enzyme-digested *E. coli* DNA or *E. coli* cells were used as controls in the Southern blot and dot blot hybridization experiments, respectively.

Lack of immunological relatedness between OmpF and OmpU. Triton X-100-solubilized outer membrane proteins of *V. cholerae* were separated by SDS-PAGE, blotted onto a nitrocellulose filter, and reacted with *E. coli* OmpF antiserum. The *E. coli* OmpF antiserum did not react with OmpU of *V. cholerae* but reacted with OmpS and a 66-kDa minor outer membrane protein (data not shown). An antibody raised against the trimeric form of OmpU cross-reacted with both monomers and trimers of OmpU but did not react with the *E. coli* OmpF.

DISCUSSION

The porins, which form water-filled channels across the outer membrane through which many antibiotics and hydrophilic compounds enter the cell, have been examined in *V. cholerae* with a view to explaining the sensitivity of these cells to a wide variety of chemicals in general and hydrophilic compounds in particular. From indirect evidence, it has been suggested that OmpT and OmpU function as porins in *V. cholerae* (13). The results presented in this report establish the pore-forming capability of these two proteins directly. By using reconstituted systems, such as liposomes, in which purified OmpT or OmpU was embedded, porin-mediated permeability was estimated quantitatively. Similar to *E. coli* OmpF and OmpC, the pore size of OmpT, the synthesis of which resembles that of OmpC with respect to the osmolarity of the growth medium, is smaller than the pore size of OmpU, which is analogous to OmpF.

Studies with purified OmpU have revealed several distinctive features of this protein compared with OmpF of *E. coli*, which apparently functionally resembles OmpU. The pore sizes of OmpU (1.6 nm) and OmpF (1.2 nm) are significantly different, and the specific activity of OmpU is 2.5 times higher than that of OmpF. The diffusion rate of a solute through the outer membrane is dependent upon, among other factors, the number of porin molecules present per cell. OmpU of *V. cholerae* constitutes about 30% of the outer membrane protein under normal growth conditions and almost 60% when grown in the absence of salt in the growth medium. The higher permeability of *V. cholerae* outer membrane might be due to the abundance of OmpU. The structure of porin molecules also dictates the rate of diffusion of a solute through the membrane. In *E. coli*, the diameter of the OmpF pore is not constant throughout the length of the channel; the middle of the channel is constructed (19). On the other hand, the diameter of *Pseudomonas aeruginosa* porin is almost constant throughout the length of the porins (21). The diameter of the pores that determines the exclusion limit of the solute as well as the rate of diffusion is that of the narrowest part of the channel. Whether the higher specific activity of OmpU compared with that of OmpF is due to a difference in the structure of the porin is not known.

The *V. cholerae* OmpU reported here is similar to that of *Rhodobacter capsulatus*, which contains three to four Ca^{2+} ions per trimer (38). Removal of Ca^{2+} ion in both cases disrupts the structure of the porin, and the pore-forming ability is lost. The Ca^{2+} present in OmpU thus forms an integral part of the protein and is essential for maintaining its structure and function. On the other hand, in the presence of EDTA, the *R. sphaeroides* porins dissociated into monomers. The monomers were active when reconstituted into proteoliposomes and produced pores with sizes comparable to those produced by the oligomer. The predominant beta-sheet structure of *R. sphaeroides* porin was retained in the EDTA-dissociated monomeric forms (37). It seems that the metal ions associated with porins of different organisms might perform different roles in the structure-function of these water-filled channels.

The OmpU of *V. cholerae* hemagglutinates human erythrocytes. Hemagglutinating activity is associated with the colonization potential of enteric pathogens, and in view of the fact that, other than Tcp, no other colonization factor has so far been identified with certainty in *V. cholerae*, it will be intriguing to investigate the potential of OmpU as a colonization factor. Preliminary studies have indicated that both OmpU and OmpT are expressed under in vivo conditions (unpublished observation).

The *ompU* gene has very little nucleotide sequence homology with *ompF* of *E. coli*. This has been reported to be true also for the *Rhodobacter* porin (19). Anti-OmpF antiserum did not cross-react with OmpU but is immunologically related to OmpS. The three-dimensional structures of the *E. coli* and *Rhodobacter* porins are similar in spite of the lack of nucleotide sequence homology (19). The absence of homology between the porins from different organisms might reflect a difference in their evolutionary origin. *V. cholerae* genes other than porin genes that have been reported so far showed significant homology with the corresponding *E. coli* genes. It is possible that the porins that are the target molecules recognized by the host immune system or bacteriocins undergo rapid changes.

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