Channel Formation by FhaC, the Outer Membrane Protein Involved in the Secretion of the Bordetella pertussis Filamentous Hemagglutinin*

(Received for publication, July 27, 1999, and in revised form, September 17, 1999)

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Many virulence factors of pathogenic microorganisms are presented at the cell surface. However, protein secretion across the outer membrane of Gram-negative bacteria remains poorly understood. Here we used the extremely efficient secretion of the Bordetella pertussis filamentous hemagglutinin (FHA) to decipher this process. FHA secretion requires a single specific accessory protein, FhaC, the prototype of a family of proteins necessary for the extracellular localization of various virulence proteins in Gram-negative bacteria. We show that FhaC is heat-modifiable and localized in the outer membrane. Circular dichroism spectra indicated that FhaC is rich in β-strands, in agreement with structural predictions for this protein. We further demonstrated that FhaC forms pores in artificial membranes, as evidenced by single-channel conductance measurements through planar lipid bilayers, as well as by liposome swelling assays and patch-clamp experiments using proteoliposomes. Single-channel conductance appeared to fluctuate very fast, suggesting that the FhaC channels frequently assume a closed conformation. We thus propose that FhaC forms a specific β-barrel channel in the outer membrane for the outward translocation of FHA.

The proper addressing of proteins to their specific cellular compartment is a fundamental process in biology and often requires complex machineries for the translocation of proteins across membranes. In the case of Gram-negative bacteria, the presence of two membranes poses a challenge for protein secretion into the extracellular medium, and several molecular strategies have been adopted to address it. Basically, they can be divided into Sec-independent and Sec-dependent pathways. During Sec-dependent secretion, exoproteins cross the cytoplasmic membrane and the outer membrane in distinct steps (1). The Sec machinery catalyzes the translocation across the cytoplasmic membrane, during which an N-terminal signal peptide is removed. Various systems exist for exoprotein translocation across the outer membrane, ranging from autosecretion (no accessory protein involved) (2) to the general secretory (or type II) pathway, which makes use of a complex machinery (1).

The whooping cough agent Bordetella pertussis secretes an array of adhesins and toxins in the course of infection of the human respiratory tract (3). Among them, filamentous hemagglutinin (FHA) 1 is a large and multifunctional adhesin secreted in remarkably large quantities (4). The mechanism of FHA secretion is of particular interest, as despite its high efficiency it requires only one specific accessory protein, FhaC. It appears to be a distinct terminal branch of Sec-dependent secretion widely distributed among Gram-negative bacteria, as a growing number of FhaC homologs are being identified that are involved in the secretion of hemolysins or adhesins (5–8).

FHA is synthesized as a preprotein whose N-terminal signal peptide is removed in the course of secretion (9, 10) and transits through the periplasmic space prior to translocation across the outer membrane (11). FHA most likely interacts in a specific manner with FhaC to reach the outer surface of the cell (12). It probably crosses the outer membrane in an extended conformation and acquires its tertiary structure once it has reached the cell surface (11).

Despite the importance of FhaC and homologs in the secretion mechanism of their cognate exoproteins, little is known on their molecular structure and function. These approximately 60-kDa proteins most probably share important structural features because they can be aligned such that 19 predicted conserved amphipathic β-strands can be identified among them.2 In addition, they all bear a C-terminal motif with a terminal phenylalanine characteristic of the OmpF/C family of porins (13). Therefore, they are hypothesized to form β-barrel channels in the outer membrane to specifically let out their cognate exoproteins (14). In this paper we report on the purification and characterization of FhaC. Circular dichroism (CD) spectra indicate that FhaC is rich in β-strands. We also provide evidence that it forms channels in proteoliposomes and lipid bilayers.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—B. pertussis strains BPSM (15), BPGR4 (ΔfhaB) (16), and BPEC (ΔfhaC) (11) and Escherichia coli UT5600 (E. coli Genetic Stock Center, Yale University, Yale, CT) were grown as described (10). pJD12 encodes Fha44 (10). pEC24 encodes functional FhaC with the E. coli OmpA signal sequence (11). pT7FCHis6 was generated as follows. After the elimination of the BamHI site in pEC24 using the Klenow enzyme on the linearized plasmid followed by religation, a new BamHI site was introduced into the first EcoRV site of fhaC by ligation of the GGAATCC linker. The annealed oligonucleotides 5’GATCCCAATATTACGACCAACCGGCCTG3’ and 5’GATCAGCCGCCCGTAATTACGACCAACCGGCCTG3’ were used to amplify the fhaC coding sequence with overlapping oligonucleotides as primers. The resulting PCR product was inserted into the BamHI site of pEC24 using T4 DNA ligase.

1 The abbreviations used are: FHA, filamentous hemagglutinin; PAGE, polyacrylamide gel electrophoresis; pS, picosecond.

2 P. Jacob-Dubuisson, J. Tommassen, and S. Guédin, unpublished data.
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CCTGTAATGGTGTAGTGTTGCTG3' were then introduced into this BamHI site, and their orientation was determined by sequencing, resulting in pFFCHis6. The fhaC-encoding XbaI-SacI fragment of pFFCHis6 was cloned under the control of the T7 promoter into pET22b, removing the PolB leader sequence of this vector (Novagen, Le Perray en Yvelines, France). The resulting plasmid, pT7FFCHis6, which codes for FhaC with a 6-His tag in the N-terminal region of the protein, was introduced into E. coli BL21(DE3)-omp5 (∆lamBompR) (17) containing a λ-borne copy of the T7 polymerase gene. For the complementation of B. pertussis BPEC by His6-tagged FhaC, pFPBPHis6 was generated as follows. pFJD163, which codes for FhaC with its own signal peptide in a plasmid vector in B. pertussis, was obtained by eliminating the Pflu site in the polylinker of pFJD16 (16) using T4 polymerase treatment of the linearized vector followed by religation. The PvuI-SacI fragment of pFFCHis6, which contains the sequence coding for most of the mature portion of FhaC including the His6 tag, was substituted for the corresponding fragment of pFJD163, yielding pFPBPHis6.

Production and Purification of FhaC—BL21(DE3)-omp5 (pET FC-His6) was grown at 37 °C in liquid LB broth to an absorbance of 1 (A_{600}) and treated with 1 mM isopropyl-1-thio-D-galactoside (final concentration) for 2 h. Cells were collected, washed in 20 mM sodium phosphate (pH 7), and resuspended in the same buffer containing 0.01 mg/ml DNase and a mixture of protease inhibitors (Roche Molecular Biochemicals, Meylan, France). Cells were broken by passage through a French pressure cell. Following a 1-h, 100,000 × g ultracentrifugation, the membrane pellet was extracted twice successively by rocking at 37 °C in the same buffer containing 1% β-octyl glucoside (Calbiochem, Meudon, France) and 2 mM EDTA for the second extraction. The second extract was subjected to chromatography on a 1.6-lm Self Pack 20 HS Pores column (Perkin-Elmer, Courtaboeuf, France) equilibrated in 20 mM sodium phosphate (pH 7) with 0.4% Elugent (Calbiochem), using a Bioaead apparatus (Perkin-Elmer). FhaC was eluted by a pulse of 300 mM NaCl in the equilibration buffer. The FhaC-containing fractions were pooled and applied onto a 1-mm HiTrap chelating column (Amersham Pharmacia Biotech, Orsay, France) equilibrated in 20 mM HEPES (pH 7.4), 0.4% Elugent, 5 mM imidazole. FhaC was eluted by a pulse of 200 mM imidazole in the equilibration buffer, aliquoted, and stored at −80 °C. FhaC was quantified as described (18), and its purity was assessed by silver staining after SDS-PAGE. To obtain a control preparation, a culture of BL21(DE3)-omp5 (pQE23) not producing FhaC was subjected to the same procedure. The absence of porins in the purified preparations was assessed by immunoblotting using an anti-porin antiserum (a gift from J. Tommassen, Utrecht, the Netherlands).

Membrane Fractionation—E. coli membrane preparation and fractionation were performed as described (19). The NADH oxidase activity of the fractions was measured as in Ref. 11. Isolation of B. pertussis outer membranes using Sarkosyl was described (10).

Protease Digestion—Protease digestion was carried out as described (20). Cells or spheroplasts were treated with 0.5 mg of trypsin for 15 min on ice or with 100 μg of proteinase K at 37 °C for 15 min. The proteins were precipitated by trichloroacetic acid at 10% final concentration, and analyzed by SDS-PAGE followed by immunoblotting with an anti-FhaC antiserum (10).

CD Spectroscopy—Purified FhaC in 20 mM sodium phosphate (pH 7), 0.4% Elugent, 5 mM imidazole was dialyzed against 20 mM sodium phosphate (pH 7), 0.4% Elugent, 200 mM imidazole was reconstituted into liposomes (9). As a positive control, 1–2 μg of outer membranes of E. coli UT5600 prepared by Sarkosyl extraction (10) were used. The negative controls included the buffer alone and a purified fraction from BL21(DE3)-omp5 (pQE32) (see above). Stachyose (666 Da) was used to establish the isosmotic point. The reconstitution of FhaC into the liposomes was verified by flotation on a sucrose gradient as described (23), followed by immunoblotting of the fractions. Swelling of the liposomes in the various isosmotic sugar solutions was monitored on a Uvikon spectrophotometer at 400 nm.

Reconstitution into Planar Lipid Bilayers—From a 0.5% solution of azolectin (Sigma, St. Quentin Fallavier, France) in hexane, virtually solvent-free planar lipid bilayers were formed by the apposition of two monolayers as described (24). The bilayer was formed on a 125-μm diameter hole in a thin Teflon film (10 μm) sandwiched between two half-glass cells and pretreated with hexadecane/hexane (1:40, v/v). The current fluctuations were recorded using a BLM 120 amplifier and stored on a DTR 1202 (Biologic, Claix, France). The stored signals were transferred to a computer and treated with Biotools software from Biologic. The electrolyte solutions were 1 M KCl buffered with 10 mM HEPES (pH 7.2), and the concentration of reconstituted FhaC was about 10^{−8} M.

Reconstitution of FhaC into Liposomes and Patch-clamp Recordings—Purified FhaC in Elugent was incubated with azolectin liposomes at a protein to lipid ratio of 1:2000 (w/w) for 30 min at room temperature before the addition of Bio-Beads SM-2 (Bio-Rad, Ivry sur Seine, France) (80 mg/ml). After 4 h of incubation at room temperature, Bio-Beads were discarded, and the suspension was centrifuged for 20 min at 357,000 × g. The pellet was resuspended in 20 μl of HEPES (pH 7.2), and aliquots were subjected to dialysis against hexadecane/hexane (1:40, v/v) to obtain giant liposomes (25). The giant proteoliposomes, collapsed in the recording chamber containing 200 mM KCl, 40 mM MgCl2, 10 mM HEPES (pH 7.2), were examined using the standard patch-clamp technique (25).

RESULTS

Subcellular Localization of FhaC—FhaC in membrane extracts of B. pertussis is partly resistant to solubilization by Sarkosyl, an indication for its outer membrane localization (10). To confirm that FhaC is associated with the outer membrane, total membranes of B. pertussis were fractionated by ultracentrifugation on sucrose gradients. However, neither flotation nor sedimentation gradients afforded a reproducible separation of the outer and inner membranes (not shown). This may reflect particular features of the B. pertussis cell envelope. We therefore performed fractionation experiments in FhaC-producing E. coli UT5600(pEC24) (11). This strain secretes an FhaC derivative, Fha44, in an FhaC-dependent manner when a compatible Fha44-encoding plasmid is present in trans, indicating that FhaC is functional (11). Membrane fractionation experiments showed that FhaC is mostly associated with the outer membrane, as evidenced by the presence of porins in the same fractions, and not with the cytoplasmic membrane, characterized by NADH-oxidase activity (Fig. 1).

FIG. 1. Subcellular localization of FhaC. Total membranes of FhaC-producing E. coli UT5600(pEC24) were subjected to sedimentation by ultracentrifugation through a 30–55% sucrose gradient. Fractions of 500 μl were collected from the bottom of the tube, and their NADH-oxidase activities (indicated by + + + +) were determined as a marker for the cytoplasmatic membrane. Even-numbered fractions were analyzed by SDS-PAGE. One gel (top panel) was stained with Coomassie Blue; the other gel (bottom panel) was subjected to immunotransfer, and the membrane was probed with an anti-FhaC antiserum. The position of the OmpFC porin is indicated. The masses (in kDa) of the molecular mass markers are shown on the left.
We then tested whether FhaC is sensitive to proteases added to intact cells. E. coli cells producing FhaC alone or in the presence of FhaI44 were treated with trypsin or proteinase K. FhaC was hardly digested under those conditions (Fig. 2), suggesting that it is embedded in the membrane or highly structured. In contrast, FhaC was substantially proteolysed after the outer membrane was permeabilized by a Tris-EDTA treatment (Fig. 2).

**Heat Modifiability of FhaC**—Because heat modifiability is a hallmark feature of many integral outer membrane proteins, we tested the heat modifiability of FhaC upon electrophoresis under semidenaturing conditions. When outer membrane extracts of B. pertussis BPSM (FhaC+, FHA+, lanes 1–4), BPGR4 (FhaC+, FHA+, lanes 5–8) or BPEC (FhaC−, FHA−, lane 9) were incubated at 20 °C for 15 min (lanes 2, 4, 6, 8, and 9) or 95 °C for 5 min (lanes 1, 3, 5, and 7) in modified Laemmli buffers containing only 0.2% SDS (lanes 3, 4, 7, 8, and 9) or 1% SDS (lanes 1, 2, 5, and 6), they were analyzed by PAGE at 4 °C without SDS in the gel and 0.1% SDS in the electrophoresis buffer. The gels were heated in steam for 8 min prior to electrotransfer, and the membrane was probed with an anti-FhaC antisera. The gels were silver-stained. The arrowhead indicates the position of FhaC. Panel B, purified FhaC was analyzed by immunoblotting with anti-porin antisera (lane 2). The outer membrane extract of E. coli UT5600 is shown in lane 1 as a positive control. The masses (in kDa) of the molecular mass markers are shown on the left of each panel.

![Fig. 2. Proteolysis of FhaC in cells.](image)

**Fig. 2.** Proteolysis of FhaC in cells. E. coli UT5600(pEC24) producing FhaC alone (odd-numbered lanes) or E. coli UT5600(pEC24, pJFD12) coexpressing FhaC and Fha44 (even-numbered lanes) were treated with trypsin (lanes 3, 4, 9, and 10), proteinase K (lanes 5, 6, 11, and 12), or mock-treated (lanes 1, 2, 7, and 8). In lanes 7–12, the outer membrane of the cells was permeabilized by a Tris-EDTA treatment prior to digestion. Proteins in all samples were precipitated with trichloroacetic acid and analyzed by immunoblotting with a purified anti-FhaC antiserum. The masses (in kDa) of the molecular mass markers are indicated on the left.

![Fig. 3. Heat modifiability of FhaC.](image)

**Fig. 3.** Heat modifiability of FhaC. Outer membrane extracts of B. pertussis BPSM (FhaC+, FHA+, lanes 1–4), BPGR4 (FhaC+, FHA+, lanes 5–8) or BPEC (FhaC−, FHA−, lane 9) were incubated at 20 °C for 15 min (lanes 2, 4, 6, 8, and 9) or 95 °C for 5 min (lanes 1, 3, 5, and 7) in modified Laemmli buffers containing only 0.2% SDS (lanes 3, 4, 7, 8, and 9) or 1% SDS (lanes 1, 2, 5, and 6). They were analyzed by PAGE at 4 °C without SDS in the gel and 0.1% SDS in the electrophoresis buffer. The gels were heated in steam for 8 min prior to electrotransfer, and the membrane was probed with an anti-FhaC antisera. **Panel A** shows the presence of FHA in the culture supernatant of an FhaC-deficient B. pertussis strain complemented with His-tagged FhaC with approximately similar efficiency (not shown). FhaC was overproduced in an ompRΔlamB E. coli strain to minimize contamination by porins (17). FhaC was extracted from membranes using β-octyl glucoside (Fig. 4A, lane 1) and subjected to a cation-exchange chromatography (Fig. 4A, lane 2) followed by a metal chelate affinity chromatography (Fig. 4A, lane 3), both performed in the presence of the nonionic detergent Elugent. Purified FhaC was devoid of OmpF and OmpC, as evidenced by immunoblotting (Fig. 4, panel B). N-terminal sequencing of the purified hetero-oligomers. However, attempts to cross-link proteins in whole cells or in membrane extracts using formaldehyde or dithio-bis(sulfosuccinimidylpropionate) yielded inconclusive results as to whether FhaC is part of oligomeric structures (not shown).

**Purification of FhaC**—To facilitate the purification of FhaC, an N-proximal polyhistidine tag was introduced into the protein. Hisω-tagged FhaC retained in vivo activity, as it secreted FHA in the culture supernatant of an FhaC-deficient B. pertussis strain complemented with His-tagged FhaC with approximately similar efficiency (not shown). FhaC was overproduced in anompRΔlamB E. coli strain to minimize contamination by porins (17). FhaC was extracted from membranes using β-octyl glucoside (Fig. 4A, lane 1) and subjected to a cation-exchange chromatography (Fig. 4A, lane 2) followed by a metal chelate affinity chromatography (Fig. 4A, lane 3), both performed in the presence of the nonionic detergent Elugent. Purified FhaC was devoid of OmpF and OmpC, as evidenced by immunoblotting (Fig. 4, panel B). N-terminal sequencing of the purified hetero-oligomers. However, attempts to cross-link proteins in whole cells or in membrane extracts using formaldehyde or dithio-bis(sulfosuccinimidylpropionate) yielded inconclusive results as to whether FhaC is part of oligomeric structures (not shown).
protein yielded the sequence Gln-Ala-Gln-Leu, showing that the signal peptide had been removed.

**CD Measurements**—Fig. 5 shows a CD spectrum of purified FhaC with a maximum ellipticity between 210 and 220 nm, characteristic of proteins having a substantial amount of β-strand structure. The β-strand, α-helix, β-turn, and random-coil contents were estimated to be 39.6, 9.3, 19.6, and 33.2%, respectively, with good precision (normalized mean root square deviation, 1.4%).

**Channel Formation by FhaC**—To investigate a channel-forming activity of FhaC, the purified protein was reconstituted into liposomes, and the rates of penetration of various sugars into these proteoliposomes were measured using a liposome swelling assay. As a positive control, the rates of swelling of proteoliposomes loaded with *E. coli* outer membrane proteins (circles). The initial rates of swelling were plotted as the percentages of the swelling rates with arabinose. The plot represents the average of three experiments.

The presence of buffer or a purified preparation obtained from non-recombinant *E. coli* using the same extraction and purification procedures as for FhaC. No swelling was observed for any of the proteoliposome preparations with stachyose (666 Da). In contrast, significant swelling of the FhaC proteoliposomes was recorded for several small sugars such as L-arabinose (150 Da), galactose (180 Da), and N-acetylgalactosamine (221 Da) (Fig. 6A). The rate of swelling was proportional to the amount of FhaC added (not shown) and inversely proportional to the size of the sugar. The specific activity of the FhaC proteoliposomes for arabinose was approximately 0.02 ΔΑ_{400} μm/μg of FhaC, which is significantly lower than that of OmpF (26). This raises the possibility that FhaC exists mostly as channels in a closed conformation, provided that most of the FhaC molecules have been reconstituted in a functional form. No swelling was observed for the negative control liposomes, whereas high rates of swelling were measured for the proteoliposomes containing *E. coli* outer membrane proteins, albeit with the smaller sugars only (Fig. 6B). This suggests that the FhaC channels are somewhat larger than those formed by the *E. coli* porins. Although it does not inhibit the in vivo activity of FhaC, we obviously cannot rule out that the insertion of the His tag may have minor effects on the properties of the channel.

Channel formation by FhaC was further studied by single-channel conductance measurements after reconstitution of the protein into planar lipid bilayers or in giant liposomes for examination by the patch-clamp procedure. Independently of the technique used, FhaC formed ion-permeable channels showing relatively brief openings at all voltages in the range of −80 to 80 mV. No threshold potential was necessary to activate the channels, and no voltage-dependent closures were observed between −80 mV and 80 mV. Fig. 7A shows recordings from the same planar bilayer at +40 mV and −40 mV in 1 M KCl. Conductance values were found to be similar (see amplitude histograms on the right of the recordings) and close to 1200 pS. Fig. 7B shows recordings from one excised patch of liposome examined by patch-clamp. The conductance values obtained in 200 mM KCl were also similar at positive and negative applied potentials. The associated amplitude histograms revealed a
main conductance step of 300 pS, in good agreement with the one already measured in 1 M KCl in planar bilayers. Indeed, the single-channel conductance of many large diffusion pores is a linear function of the specific conductance of the bulk aqueous phase (27). The two amplitude histograms obtained from recordings of FhaC in liposomes revealed another step of conductance estimated to 30–50 pS. It was also observed in planar bilayer experiments, but it was not so easily detected because of the higher noise and lower time resolution of this latter technique compared with the patch-clamp technique.

**DISCUSSION**

Exoprotein secretion in Gram-negative bacteria involves one of several families of accessory proteins thought to form protein transport channels that span the outer membrane (28). No high resolution structure is available yet for any of them. Secretins form one such family (28). They are outer membrane components of the type II and type III protein secretion machineries and form ring-shaped, highly stable oligomeric channels (29–31), the size of which would be compatible with the transport of folded proteins. Molecular ushers represent another family. The PapC usher involved in P pilus biogenesis is a ring-shaped hexamer with an inner diameter of 2–3 nm and has also been shown to form sugar-permeable channels in artificial membranes (32). The trimeric TolC represents a third kind of protein transport channel in the outer membrane (33). By its primary structure and the properties reported in this study, FhaC appears to represent a yet distinct family of protein transport channels in the outer membrane of Gram-negative bacteria.

FhaC is localized in the outer membrane, as shown by its heat modifiability and cell fractionation experiments. CD spectra indicate that it is rich in β-strands. The simplest structural model for FhaC would be that of a β-barrel in the outer membrane, with a central opening for FHA outward translocation. His6-tagged FhaC forms sugar-permeable channels in liposomes and ionic channels in planar lipid bilayers and in giant liposomes examined by the patch-clamp technique. These ion channels are characterized by short lifetimes of a few hundred milliseconds and conductance values in 1 M KCl similar to that of the OmpF monomer (34). However, the FhaC channel lifetimes significantly differ from those of E. coli porins, which are characterized by slow transitions of current. This could explain the low specific activity of FhaC for arabinose in liposome swelling experiments, when compared with liposomes doped with E. coli porins. Such a flickering behavior in planar lipid bilayers is not unprecedented for outer membrane proteins, as it has been observed with TolC (35), the FhaC homolog ShIB (14), and recently with a phage-encoded protein (pIV) of the secretin family (31). The high conductance values of the pIV ion channels in planar lipid bilayers suggest large diameter pores, necessary to accommodate a phase secreted through the outer membrane of the bacterial host. In contrast, the lower conductance values for FhaC channels are in agreement with the notion that FHA crosses the outer membrane in an extended conformation (11).

We cannot draw a firm conclusion as to whether FhaC is part of oligomeric structures in the envelope. Our results suggest that FhaC may be part of rather labile complexes, the biological relevance of which is unknown. However, no protein of the Fhac family has been shown to form oligomers, and ShIB has been reported to be monomeric (14). In addition, several other β-barrel outer membrane proteins such as OmpA, FhuA, and BtuB appear to be monomeric (26, 36–38).

It is unknown whether FhaC is gated in vivo. Perhaps its low natural abundance and mostly closed state make gating unnecessary. We have observed some conformational change in FhaC upon FHA secretion. The interaction of FHA with FhaC in the course of FHA secretion could lead to the opening of the FhaC channel or to the stabilization of an open state. Thus, we need to characterize the specific molecular interactions between FHA and FhaC and the forces that drive FHA translocation. FhaC might simply provide for the specific, facilitated outward diffusion of FHA, somewhat similar to channels such as those formed by LamB used for the inward diffusion of small solutes across the outer membrane (39).

**Acknowledgments**—We thank E. Fort for photography, D. Thanassi for the liposome swelling protocol, H. Drobecq for N-terminal sequencing, and H. Koebnik for BL21(DE3)omp5. We are grateful to J. Dubuisson for his comments on the manuscript.

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