Channel Properties of TpsB Transporter FhaC Point to Two Functional Domains with a C-terminal Protein-conducting Pore

Received for publication, August 3, 2005, and in revised form, October 12, 2005 Published, JBC Papers in Press, November 12, 2005, DOI 10.1074/jbc.M508524200

Albano C. Méli1,2, Hélène Hodak1,3, Bernard Clantin4, Camille Locht4, Gérard Molle4, Françoise Jacob-Dubuisson5,4 and Nathalie Saint1,4

From the 1UMR 5048 CNRS, U554 INSERM, Centre de Biochimie Structurale, 29 Rue de Navacelles, 34090 Montpellier Cedex, 2INSERM U629 and 3UMR 8525 CNRS, Institut de Biologie de Lille, Institut Pasteur de Lille, 1 Rue Calmette, 59019 Lille Cedex, France

Integral outer membrane transporters of the Omp85/TpsB superfamily mediate the translocation of proteins across, or their integration into, the outer membranes of Gram-negative bacteria, chloroplasts, and mitochondria. The Bordetella pertussis FhaC/FHA couple serves as a model for the two-partner secretion pathway in Gram-negative bacteria, with the TpsB protein, FhaC, being the specific transporter of its TpsA partner, FHA, across the outer membrane. In this work, we have investigated the structure/function relationship of FhaC by analyzing the ion channel properties of the wild type protein and a collection of mutants with varied FHA secretion activities. We demonstrated that the channel is formed by the C-terminal two-thirds of FhaC most likely folding into a β-barrel domain predicted to be conserved throughout the family. A C-proximal motif that represents the family signature appears essential for pore function. The N-terminal 200 residues of FhaC constitute a functionally distinct domain that modulates the pore properties and may participate in FHA recognition.

Targeting and translocation of soluble and integral membrane proteins to the ad hoc subcellular compartments are essential for cell function and organelle biogenesis. Transport of proteins across membranes and their insertion into membranes are typically mediated by proteinaceous transmembrane complexes, and some of these pathways have been conserved throughout evolution (1). With their complex envelope structure, Gram-negative bacteria and eukaryotic organelles face similar challenges for the translocation of proteins (2–4). In the outer membrane of Gram-negative bacteria, specific membrane-embedded β-barrel proteins are essential components of protein transport machineries (5). Similarly, polypeptide-transporting β-barrel proteins are also found in the outer membranes of eukaryotic organelles of prokaryotic origin such as chloroplasts and mitochondria (6, 7).

The recently revealed Omp85/TpsB superfamily of outer membrane proteins is dedicated to protein transport in most major kingdoms of life, although no members have been identified in Archaebacteria yet (8, 9). TpsB transporters are found in two-partner secretion systems, developed by Gram-negative bacteria for the secretion of large “TpsA” proteins destined to the cell surface or the milieu and serving mostly as virulence factors (10). The other members of the Omp85/TpsB superfamily are transporter proteins included in large hetero-oligomeric complexes, such as the Toc75 homologs in chloroplasts, the Tob55/Sam50 homologs in mitochondria and the Omp85 homologs in Gram-negative bacteria. These transporters mediate inward protein transport across the chloroplast outer membrane and assembly of β-barrel proteins into the outer membrane of mitochondria and Gram-negative bacteria, respectively (11–15).

Although their sizes and origins vary, the proteins of the TpsB/Omp85 superfamily are phylogenetically related and have been postulated to derive from a common ancestor, probably a simple prokaryotic channel (4, 16, 17). They are all predicted to be composed of a number of amphipathic β-strands, most likely forming a transmembrane β-barrel, and channel activity was demonstrated for several of them (12, 16, 18–21). Their sequences share conserved C-proximal sequence motifs of unknown function (8, 9, 17). Despite their implication in critical biological processes such as membrane biogenesis and secretion of virulence factors, none of these proteins has been characterized structurally so far, and the currently available topology models are somewhat conflicting (13–15, 18, 22–24).

Bordetella pertussis secretes its major adhesin, FHA, via a TpsB family member, the outer membrane transporter FhaC (25). A topology model for FhaC was proposed earlier, based on sequence alignments of several TpsB proteins, predicting 19 amphipathic β-strands linked by large surface loops and periplasmic turns (24). The C-terminal portion of this model was reasonably well validated by an epitope insertion method, whereas the N-terminal moiety has remained significantly less well defined (24). FhaC was shown to form ion-permeable channels in artificial membranes, although the relationship between this pore activity and the FHA-secreting activity of FhaC has not been established (21).

A collection of mutant FhaC proteins each carrying a two-residue insertion at a specific sequence position were generated previously (24). In this work, a number of these mutant FhaCs with varied FHA secretion activities were purified, and their electrophysiological characteristics were studied at both macroscopic and single-channel levels and compared with those of the wild type (WT) protein. We have thus established that the C-terminal two-thirds of FhaC participate in channel formation, and its first 200 residues most likely form a functionally distinct domain. The results also suggest that the ion channel corresponds to the protein-conducting pore.

* This work was supported in part by Ministère de l’Éducation Nationale and Recherche and Technologie Grant ACI BCM52004. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This on-line version of this article (available at http://www.jbc.org) contains a Table and Figs. S1 and S2.

1 Both authors contributed equally to this work.

2 Recipient of a predoctoral fellowship from the Ministère de l’Éducation Nationale and Recherche and Technologie.

3 Recipient of a joint predoctoral fellowship from the Re´gion Nord Pas-de Calais and the Ministère de l’Education Nationale.

4 Both authors should be considered as senior authors.

5 Researcher for the CNRS. To whom correspondence should be addressed. Tel: 33-320-87-11-55; Fax: 33-320-87-11-58; E-mail: francoise.jacob@ibl.fr.

6 The abbreviations used are: WT, wild type; TEV, tobacco etch virus.
Channel Properties of TpsB Transporter FhaC

EXPERIMENTAL PROCEDURES

Plasmid Construction—pFJD118 was constructed as follows. pFcc3 (24) was linearized by restriction with BamHI, which cleaves at a unique site positioned after the third codon into the sequence of mature FhaC. The oligonucleotides 5′-GATCCCATACCAACCGG-3′ and 5′-GATCCATACCAACCGG-3′ were annealed and inserted into BamHI-restricted pFcc3. The orientation of that linker was verified by sequencing, and the resulting plasmid was called pFcc3-His6. The latter plasmid was restricted with XbaI and SacI, and the fhaC-containing DNA fragment was inserted into the same sites of pET24d (Novagen), resulting in pFJD118. To prepare the pFJD118 derivatives harboring fhaC each with a given insertion, pFJD118 was restricted with PstI (which cleaves a few nucleotides 3′ of the position of the BamHI site in fhaC) and SacI (which cleaves after the termination codon of fhaC). The PstI-SacI fragments prepared from the corresponding pFccx, pMycx, or pTEVx plasmids (24), each coding for FhaC with a particular insertion (or deletion), were exchanged for the WT fragment of pFJD118. The resulting plasmids were called pT7FcxB (where x represents the position of the codon with a BamHI insert), pT7FcxM (for a Myc-encoding insert at codon x), pT7FcxT (for a tobacco etch virus (TEV) cleavage site encoding insert at codon x), or pT7FcxΔ3-206 (with a deletion between codons x and y). The corresponding amino acid sequences of the mutants are shown in the supplemental Table.

To construct pFJD150, the plasmid encoding the C-terminal truncate FhaC-A3–206, pFcc206 was restricted with BamHI and HindIII, and the resulting fragment coding for the last 349 residues of FhaC was inserted into a pFJD118 derivative called pFJD138 in replacement of its own BamHI-HindIII fragment. pFJD138 is similar to pFJD118 except that there is a BamHI site only 3′ of the His6 tag-coding linker, but not in the 5′ position. The resulting pFJD150 encodes the C-terminal truncate of FhaC with a His6 sequence located three residues after the signal peptide cleavage site.

Expression and Purification of Wild Type and Mutant FhaC Proteins—The WT and mutant FhaC proteins were produced and purified, as described previously (21) from Escherichia coli BL21(DE3)-ompS (26) carrying each of the pT7Fc plasmids, with the following modifications. After harvesting the membrane fractions by ultracentrifugation, two steps of extraction were performed successively with 0.8 and 1.5% β-octyl glucoside. The second detergent extract was chromatographed as described previously (21), except that 1% Elugent was used in the buffers rather than 0.4%. The metal-chelate chromatography was performed with 400 mM imidazole in the elution buffer.

Secretion Assay—E. coli UT5600 bacteria harboring pFJD12 (coding for Fha44, a C-terminally truncated version of FHA whose secretion properties parallel those of FHA (27)) and each of the plasmids of the pFccx series (or pTEVx or pMycx) were grown as described previously (24, 28). The culture supernatants were harvested following a 2-h period of induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside and were subjected to electrophoresis in polyacrylamide gels in the presence of SDS. The proteins were then transferred onto a nitrocellulose membrane, and immunodetection was performed using a mixture of two monoclonal antibodies raised against FHA, F4 and F5 (29). To compare the amounts of FhaC produced by those strains, bacteria were harvested from liquid cultures at mid-exponential phase, washed in 20 mM Hepes (pH 7), and resuspended in 1/10 volume of the same buffer. Equal amounts of bacteria were used for all strains. They were broken by...
Channel Properties of TpsB Transporter FhaC

passages in a French press, and the clarified lysates were subjected to a 1-h ultracentrifugation at 100,000 × g to pellet the membranes. The pellets were resuspended in 20 mM Hepes (pH 7), and membrane aliquots from each strain were analyzed by SDS-PAGE and immunoblotting using anti-FhaC antibodies (24). The amounts of Fha44 and FhaC in all strains were determined by densitometry scanning of immunoblots. The secretion activities were calculated by determining the ratios Fha44/FhaC and setting the secretion activity of the strain producing WT FhaC to 100%.

Planar Lipid Bilayer Recordings—From a 0.5% solution of azolectin (Sigma) in hexane, virtually solvent-free planar lipid bilayers were formed by the apposition of two monolayers as described (21). The membrane was formed over a 125–200-µm diameter hole in a thin Teflon film (10-µm) sandwiched between two glass cells pretreated with hexadecane/hexane (1:40, v/v). The cis- and trans-compartments contained 1 M KCl buffered with 10 mM Hepes (pH 7.4). 0.5–5 µl of purified FhaC (initial concentration of 0.2–0.5 mg/ml) were diluted 100-fold in 1% octyl-polyoxyethylene and added to the cis-compartment. The measuring cell was connected with an Ag/AgCl electrode at the cis-side, and the electrode on the so-called trans-side was grounded. All experiments were performed at room temperature.

In macroscopic conductance experiments, the doped membranes were subjected to slow voltage ramps (10 mV/s), and transmembrane currents were fed into an amplifier (BBA-01, Eastern Scientific, Rockville, MD). Current-voltage curves were analyzed with the SCOPE software (Bio-Logic, Clai, France).

In single-channel recordings, currents were amplified and potentials were applied simultaneously by a patch clamp amplifier (RK 300, Bio-Logic). Single-channel currents were monitored using an oscilloscope (TDS 3012, Tektronix, Beaverton, OR) and stored on a CD recorder (DR 200, Bio-Logic) for off-line analysis. CD data were then analyzed by the WinEDR (Bio-Logic) and Clampfit (Axon Instruments) software. Data were filtered at 1 kHz before digitizing at 11.2 kHz for analysis.

TEV Protease Digestion of FhaC-150T Mutant—Purified FhaC-150T diluted in 0.05% dodecyl maltoside was treated with TEV protease (AcTEV Protease, Invitrogen) in 40 samples of 20 µl each. The digestion was performed overnight at 30 °C in 50 mM Tris-HCl (pH 8.0) 0.5 mM EDTA, 1 mM dithiothreitol, with 6 units of AcTEV protease (10 units/µl) in each reaction tube. The ratio of TEV protease to FhaC-150T was 1:1. The samples were analyzed by SDS-PAGE.

RESULTS

Choice of the Mutant FhaC Derivative—To study the relationship between the channel properties of FhaC and its secretion activity, 20 different mutants were selected from a collection generated previously by random insertion of 6-mer BamHI linkers into the fhaC sequence (24). The corresponding mutant FhaCs contain each a 2-residue insertion at a given position in the protein (Fig. 1A and supplemental Table). Four FhaC derivatives with longer c-Myc insertions (16 residues), FhaC-125M, FhaC-260M, FhaC-434M, and FhaC-503M, were included in the analysis, as well as two additional FhaC variants, FhaC-3–26 and FhaC-3–221–228, each carrying a small deletion in predicted transmembrane domains. The levels of production of the FhaC mutant proteins were determined by immunoblot analyses of total membrane fractions of the recombinant strains using anti-FhaC antibodies (24) (Fig. 1B). In parallel, we assessed Fha44 secretion by immunoblot analyses of nonconcentrated culture supernatants of these recombinant strains using anti-FHA antibodies (Fig. 1B). The secretion activities were obtained by calculating the Fha44/FhaC ratio for each strain (Table 1). The FhaC mutant proteins secreted Fha44 with widely varied efficiencies (Table 1). Altogether, the levels of Fha44 secretion were found to depend essentially on the positions of the mutations rather than on the abundance of the mutant FhaC proteins, as observed earlier (24) (Fig. 1B and Table 1). Of note, the c-Myc insertion immediately after residue 125, although predicted to interrupt a transmembrane strand, did not affect the production of FhaC or its ability to secrete Fha44 in vivo (Fig. 1B).

We then sought to purify each of the mutant proteins in order to analyze their pore properties in lipid bilayers. To this end, WT FhaC as well as each of the mutant proteins were overproduced in a porin-deficient E. coli strain, BL21(DE3)-omp5, and the corresponding proteins were purified. Under those conditions, most mutant FhaCs were obtained at reasonable yields. However, FhaC-243B was obtained in very low amounts, whereas FhaC-33B could not be extracted from the membranes, most likely because it was significantly perturbed by the insertion. FhaC-33B was thus not considered further.

Characterization of WT FhaC in Artificial Lipid Bilayers; Macroscopic I/V Curves—In a previous study, we reported that FhaC formed ion-permeable channels showing relatively brief openings and with conductance values around 1200 pS in 1 M KCl (21). In the present study, we looked at ion channel properties of FhaC by recording macroscopic current-voltage (I/V) curves (Fig. 2A). In this configuration, up to a hundred channels are incorporated and submitted to slow voltage ramps at relatively high protein concentrations. Macroscopic I/V curves are useful to screen the functional properties of potential channel formers in lipid bilayers and allow relatively rapid comparisons between related proteins, i.e. mutants of FhaC.

The I/V curves obtained after addition of FhaC at a final concentration around 100 ng/ml to the cis-side of azolectin bilayers submitted to

### TABLE 1

| Mutation     | Secretion activity of mutant* | Channel properties |
|--------------|-------------------------------|--------------------|
|              |                               | Macroscopic I/V curve | Conductance* |
| 150B         | 117                           | WT-like            | ρS          |
| 125M         | 109                           | WT-like            | ND          |
| 532B         | 100                           | WT-like            | 1230        |
| Δ3–26        | 98                            | WT-like            | 800–1000    |
| Δ221–228     | 86                            | Affected           | 850         |
| 367B         | 81                            | WT-like            | 1050        |
| 93B          | 77                            | WT-like            | 1240        |
| 503M         | 76                            | WT-like            | ND          |
| 395B         | 59                            | WT-like            | 1115        |
| 108B         | 59                            | Affected           | 930         |
| 260M         | 32                            | Affected           | ND          |
| 462B         | 3.3                           | Affected           | NC          |
| 342B         | 2.6                           | WT-like            | 1280        |
| 434B         | 1.5                           | Affected           | NC          |
| 243B         | 0                             | Affected           | 900–3000    |
| 307B         | 0                             | Affected           | 350         |
| 434M         | 0                             | NA                 | NA          |
| 495B         | 0                             | Affected           | NC          |
| Δ3–206       | ND                            | Affected           | 200–900     |

* Secretion activities correspond to the levels of Fha44 secreted in the supernatants corrected by the levels of FhaC produced by the recombinant strains. They were obtained by determining the ratios of the intensities of the Fha44 and FhaC bands on immunoblots, with the secretion activity of the strain producing WT FhaC set to 100%. The activities shown in the table correspond to a representative experiment. The mutant FhaCs were sorted in decreasing order of activities.

* Conductances were determined in 1 M KCl using single channel recordings. NC indicates not calculated because signals were too noisy; ND indicates not determined; NA indicates no activity.
slow triangular voltage ramps (10 mV/s) were asymmetric, with the apparition of a hysteresis in the negative quadrant (Fig. 2A). This result indicates that structurally asymmetric FhaC molecules, as suggested by secondary structure predictions, insert in a preferred orientation into lipid bilayers. However, the absolute orientation of the molecules could not be determined from these current-voltage recordings. The I/V curve in Fig. 2A exhibits a linear part from +100 mV to around −60 mV, indicating a voltage-independent conductance of FhaC channels in this voltage range, whereas a curve bending appeared from −60 to −100 mV in the increasingly negative voltage branch. This latter observation suggests the following. (i) The conductance characteristics of FhaC are dependent on the membrane potential above a threshold voltage (around −60 mV). (ii) A conformational change of FhaC channels, such as a displacement of loops, may appear at this particular voltage, which results in higher ion-conducting pores as observed from increasing currents recorded above this potential. It is noteworthy that the falling voltage branch in the negative part of the I/V curve did not present a curve bending as observed in the corresponding rising voltage branch. This indicates that the channels might not switch back to their initial conformation during the relaxation process at negative voltages.

Characterization of Wild Type FhaC in Artificial Lipid Bilayers; Single-channel Measurements—In order to characterize further the FhaC channels and to confirm that the asymmetric properties in ion conduction observed from the I/V recordings were an intrinsic property of membrane-inserted FhaC, we also examined the influence of membrane potential on conductance in single-channel experiments. To this end, very small amounts of FhaC molecules were introduced into the cis-compartment bathing the lipid bilayer (final concentration around 0.1 to 1 ng/ml). Fig. 2B shows ionic currents through a single FhaC channel recorded at different membrane potentials. The current increased with increasing voltages between +40 and +80 mV, and the conductance value (about 1200 pS; see the amplitude histogram on Fig. 2C) remained stable, indicating that no voltage-dependent conductance occurred in this potential range. In contrast, the conductance characteristics of FhaC changed at negative voltages. Although altered conductance could not be well visualized at −40 mV, current recordings at −60 mV became slightly noisy and revealed that the FhaC channel occasionally displayed switches into substrates. This behavior became more pronounced at increasing negative potentials until the channel conductance appeared very noisy. Fig. 2D shows current recorded through an FhaC channel at repetitive pulses of +60 and −60 mV. The appearance of noisy currents during negative pulses is a consequence of applied negative voltages, because noisy channels did not persist when potentials were switched back to positive polarity. The increasing noise at high negative potentials could be because of an asymmetric distribution of positive and negative charges across the membrane combined with a conformational mobility of parts of the protein, as described earlier for TolC and engineered variants of LamB (30, 31). The single-channel measurements thus confirm that the FhaC channels display

FIGURE 2. Electrophysiological behavior of WT FhaC. A, I/V curves between −100 and +100 mV. The arrows indicate the direction of the applied voltage ramp. B, single-channel recordings at positive and negative voltages. C, amplitude histogram of the single-channel recordings at +60 mV. D, single-channel recordings at +60 mV and −60 mV. The dashed lines represent the zero current level.
asymmetric conductance properties depending on the sign of the applied potential.

**Effect of Mutations on Ion Conduction by FhaC**—To study the influence of mutations on the ion channel properties of FhaC, we first looked at the current-voltage curves of the mutant proteins as determined by the application of triangular voltage ramps in 1 M KCl. This method allowed us to compare the electrophysiological properties of the mutants with the signature of WT FhaC established from its I/V recordings. In addition, to extend these measurements and to examine the conductance values of mutant proteins, single-channel analyses were also performed on most FhaC derivatives.

The channel activities of several FhaC mutants harboring short insertions in two C-proximal sequence motifs conserved in the Omp85/TpsB family and predicted to be involved in the β-barrel fold (8) are displayed in Fig. 3. Among them, FhaC-434B, -462B, and -495B exhibited I/V recordings significantly different from those of the WT protein (Fig. 3A). These I/V curves were less asymmetrical than that of WT FhaC and did not display the hysteresis in the negative quadrant. These differences in channel behavior were confirmed by single-channel analyses of the mutants. Reconstitution of FhaC-434B, -462B, and -495B in planar lipid bilayers revealed a noisy membrane conductance without any well-defined single channels at negative and positive potentials (Fig. 3B). Thus, from these measurements, we were able to establish a good correlation between the loss of the hysteresis of I/V plots and the strongly affected single channel activity observed in bilayers. These results indicate unambiguously that the channels formed by these derivatives are less stable than those formed by WT FhaC. Of note, it was necessary to apply a membrane potential superior to 100 mV in order to detect some current fluctuations with FhaC-434B. This observation suggested that FhaC is considerably affected by the two-residue insertion immediately following residue 434 in the predicted loop L8. Moreover, a c-Myc insertion in the same position, which represents a 16-amino acid residues insertion, completely abolished the channel activity of FhaC, supporting the critical role of this position in channel formation (data not shown; see Table 1). The inability of these mutants to form stable channels might account for the absence of detectable secretion of Fha44 observed in vivo (Fig. 1B).

In contrast to the mutations -434B, -462B, and -495B, two insertions located after residues 503 and 532 in the second conserved C-proximal motif hardly affected Fha44 secretion in vivo. The I/V recordings of FhaC-503M were found to be similar to those of WT FhaC (Table 1), suggesting that this long insertion does not alter channel activity. Similarly, FhaC-532B displayed I/V recordings similar to those of the wild type protein (Fig. 3A). In single-channel measurements, the latter derivative exhibited membrane conductance fluctuations of 1230 pS in 1 M KCl that represent rapid closings and openings of one channel, much like WT FhaC (Fig. 3B and Table 1). This second motif thus appears much less critical to the channel properties of FhaC.

To determine the contribution of the N-terminal region of FhaC to channel formation, the pore-forming properties of several insertion mutants in this domain were measured in planar lipid bilayers. The channel activities of two such FhaC derivatives presenting only slightly altered abilities to secrete FHA in vivo are displayed in Fig. 4. FhaC-93B and -150B exhibited asymmetric I/V recordings in 1 M KCl, with a hysteresis in the negative quadrant as observed for WT FhaC. Most interestingly, in the positive quadrant the rising and falling branches of the I/V curves did not superimpose, as observed for the WT FhaC, but these two branches formed a very small hysteresis. The single-channel
recordings of both mutants showed current transitions with conductance values close to that of WT FhaC, as determined from the current amplitude histograms (Table 1; 1240 and 1180 pS for FhaC-93B and -150B, respectively). Among the other mutants in the 200 first residues, FhaC-125M, with a c-Myc insertion in a predicted transmembrane β-strand, also displayed a WT electrophysiological signature according to its I/V recordings (Table 1), similarly to FhaC-93B and -150B. These results suggest that the positions 93, 125, and 150 are not involved in channel formation.

Other data indicated nevertheless that the N-terminal domain influences the channel properties of the protein. An FhaC derivative with a short insertion following residue 108, FhaC-108B, displayed a slightly modified I/V recording compared with WT channels and noisy single channels with a somewhat reduced conductance value (Table 1 and supplemental Fig. S1). Slightly altered electrical properties were also obtained with FhaC-Δ3–26, containing a 24-residue deletion in the N-proximal part of the protein (Table 1 and supplemental Fig. S2). Altogether, analysis of the pore properties of mutants in the N-terminal domain of FhaC showed no drastic change in channel activity. Collectively, these data support the idea that although the N-terminal region of FhaC is not essential for channel formation, it might participate in its modulation.

To investigate the central portion of the protein and to test further the validity of the proposed topology (24), pore properties of other FhaC mutants with mutations between the N-terminal region and the conserved C-proximal sequence motifs were examined in planar lipid bilayers. The reconstitution in membranes of FhaC-Δ221–228, in which the major part of predicted loop L3 is deleted, resulted in modified I/V recordings with a loss of the typical hysteresis of WT channels. Furthermore, single-channel measurements revealed very fast conductance fluctuations around 850 pS in 1 M KCl (Table 1 and supplemental Fig. S2). FhaC-243B harboring a short insertion in a putative transmembrane β-strand was significantly more affected. It displayed a strongly altered channel behavior characterized by nonasymmetric I/V curves and heterogeneous conductance values ranging from 900 to 3000 pS (Table 1 and supplemental Fig. S1). It should be noted that the purification yields of that FhaC derivative were very low, suggesting an altered conformation, and it was incapacitated for FHA secretion. Collectively, these data reinforce the prediction that this region represents a bona fide transmembrane segment of the β-barrel domain.

A modified channel behavior was also observed for FhaC-260M, with a c-Myc epitope insertion in loop L4, as well as for FhaC-307B, with a two-residue insertion in loop L5 close to the membrane (Table 1 and supplemental Fig. S1). FhaC-307B exhibited a burst-like activity rather than well defined channels, and its conductance value was strongly reduced (around 350 pS in 1 M KCl). This behavior correlated with the inability of that mutant protein to secrete FHA secretion in vivo, both of which strongly argue that residue 307 is part of a region participating in channel formation as predicted in the model. As for FhaC-260M, its altered channel activity might be due to the destabilization of L4 after the insertion of the 16-residue c-Myc epitope, because FHA secretion in vivo was affected by that long insertion but not by a shorter two-residue insertion in the same position (24).

Two FhaC derivatives harboring short insertions in other predicted surface-exposed loops, L6 (FhaC-342B) and L7 (FhaC-395B), were tested similarly. Both displayed WT-like pore activities, as documented by I/V plots and single-channel conductances of 1260 and 1115 pS in 1 M KCl for FhaC-342B and FhaC-395B, respectively (Table 1 and supplemental Fig. S1). These data are compatible with the predicted localization of these residues in loop regions of the protein, and they suggest that these loops are not crucial in regulating the channel behavior. However, the FhaC-342B mutant displayed a much reduced ability to secrete FHA, suggesting a role of this loop for secretion, although not related to channel formation.

The pore properties of FhaC-367B were also investigated because it is one of the few FhaC derivatives with an insertion in a predicted periplasmic turn of the β-barrel. Notably, all four insertion mutants in periplasmic turns were able to secrete Fha44 in vivo (24), arguing against those regions having an active role in that process. Accordingly, the study of the channel properties of FhaC-367B did not reveal any important change (Table 1 and supplemental Fig. S1), confirming that it is not crucially involved in the channel properties.

*Pore-forming Properties of a C-terminal Truncate—*The electrophysiological data thus suggest that FhaC constitutes two functional domains, with the channel-forming β-barrel domain corresponding to the last 350 or 400 residues of the protein. To confirm this hypothesis,
Channel Properties of TpsB Transporter FhaC

DISCUSSION

FhaC belongs to a superfamily of protein transporters whose structure remains poorly defined except that they are all predicted to fold into transmembrane β-barrels. These proteins form ion-permeable channels in lipid bilayers (12, 16, 18–21, 32). In an attempt to functionally dissect FhaC, we analyzed the ion-conducting properties of the WT protein and a collection of mutants with varied abilities to secrete FHA. The profile of I/V recordings of WT FhaC is quite characteristic and can thus be considered its electrophysiological signature, allowing us to make comparisons between WT and mutant proteins. Single-channel measurements were also used as a complementary means to characterize the FhaC mutants.

The asymmetric behavior of the FhaC channels in response to positive- and negative-applied voltages indicates that the protein inserts into the bilayer in a defined orientation, as observed earlier with other β-barrel proteins (19, 33–35). For instance, LamB was inserted in lipid bilayers with large extra-membrane loops protruding in the compartment to which the protein was added (35). The initial, porin-like model of FhaC proposed essentially long extracellular loops and short periplasmic turns (24) (see Fig. 1A). However, the data obtained in this work have now allowed us to reassess that model. In particular, it is likely that a larger portion of the protein is oriented toward the periplasm to ensure the specific recognition of incoming FHA by FhaC on the periplasmic side of the outer membrane.

Each of the mutants studied in this work contained an insertion or a deletion at a defined position in the protein, with distinct consequences on the channel and FHA secretion activities of FhaC. Primarily two categories of behaviors were observed. The mutant proteins with no channel activity, such as FhaC-434M, or with strongly altered channel properties, i.e. affected macroscopic I/V curves and strongly altered conductance values such as FhaC-243B, -307B, -434B, -462B, and -495B, were also markedly deficient for the secretion of FHA. Furthermore, several mutants with WT-like or slightly altered channels, including FhaC-Δ3–26, -93B, -108B, -125M, -150B, -Δ221–228, -367B, -395B, -503M, and -532B, secreted Fha44 quite efficiently. These results suggest that the ion channel might be the protein-conducting pore. However, it is noteworthy that FhaC-342B, defective for secretion although its pore properties appeared normal, is clearly not fitting in any of the two categories of mutants. This particular behavior suggests that the link between the channel and secretion activities of FhaC may be more complex. It most likely reflects the fact that FhaC is not only the translocation channel of FHA but is also involved in other steps of the secretion process, including the specific recognition of its partner.

 Globally, mutations in the central and C-terminal portions of the protein were more detrimental to both FHA secretion and channel activity than those in the first 150 residues, which had milder effects on the FhaC channels. Mutations strongly affecting the channel properties correspond to insertions within, or very close to, predicted transmembrane β-stands, including 243B, 307B, 434B, 462B, and 495B, in agreement with the topology model for the C-terminal two-thirds of FhaC. In marked contrast, the channels formed by FhaC-125M, carrying a 16-residue insertion predicted to occur in the midst of a transmembrane β-strand, were not affected. This argues that the N-proximal part of the FhaC topology model needs to be revised, in particular regarding the first predicted transmembrane β-strands.

Starting from residue 243 located in a predicted transmembrane β-strand, several insertions drastically affected both FHA secretion and channel activities. In particular, the penultimate surface-exposed loop L8 appeared essential to both activities. Of note, L8 was shown previously to undergo a conformational change upon FHA secretion (24),
suggesting its functional importance. Most interestingly, this region is part of a highly conserved motif identified in the Omp85/TpsB superfamily (8). Our data emphasize that it assumes important functional properties related to the pore-forming activity of the protein.

That the C-terminal 350 residues of FhaC were necessary and sufficient for channel formation was confirmed by the ability of a C-terminal truncate of FhaC lacking the first 200 residues to make pores. The electrophysiological properties of these channels were noticeably different from those of WT FhaC, indicating that the N-terminal region modulates the pore properties of the C domain. If the channel-forming β-barrel domain of FhaC is initiated after the first 200 residues, then it is composed at the maximum of 16 β-strands according to the predicted topology. It should be noted that the N-terminal region of FhaC has a significantly lower content of aromatic residues (6.5%) than the rest of the protein (11%), which is consistent with the proposed division of FhaC into a C-terminal β-barrel forming domain and a distinct N-terminal domain.

What are the functions of this N domain? First, it appears to modulate the channel activity of the β-barrel domain, possibly together with one or several surface-exposed loops of FhaC. Another important function might be the molecular recognition of FHA. Several insertions within the region encompassing residues 90–170 were shown to affect strongly FHA secretion (24), emphasizing the functional importance of that region. Furthermore, it is very likely that the N domain is at least in part oriented toward the periplasm, as indicated by epitope insertion data showing that most of it is not surface-exposed (24) and by our current results that the first transmembrane strands might not have been predicted correctly. This orientation would be consistent with a role in FHA recognition as an initial, periplasmic step of secretion. In contrast, the prediction that the periplasmic turns linking the strands of the β-barrel domain of FhaC could serve as docking site(s) for FHA, based on the topology model, was not borne out by the finding that insertions in these turns had no effect on FHA secretion in vivo (24). In agreement with this, in the Serratia marcescens TpsB transporter ShlB, which both secretes and activates its hemolysin partner ShlA, mutations leading to secretion of nonhemolytic ShlA map precisely to the N-proximal region, proposed by these authors to chaperone and activate ShlA (36). It should be noted that the N-terminal region of TpsB proteins includes a putative POTRA motif hypothized to be involved in protein-protein interactions (37). Altogether, these observations corroborate our hypothesis that the specific recognition of TpsA proteins by their TpsB transporters may involve that region. We have demonstrated recently specific interactions between FhaC and FHA,7 and the mapping of these interactions is in progress.

Thus, we propose that FhaC is made of two functional domains, with the C-terminal two-thirds forming the protein-conducting β-barrel channel and the N-terminal region participating in substrate recognition and in channel modulation. The functional subdivision of the Omp85/TpsB proteins into a domain likely dedicated to protein-protein interactions and a C-terminal channel-forming β-barrel domain is consistent with phylogeny. In fact, although the C-terminal moieties of all the Omp85/TpsB family members are clearly homologous and predicted to contain amphipathic β-strands, their N-terminal moieties are much more diverse, probably reflecting adaptations to their particular functions (38). A two-domain model was recently proposed for Omp85, with the N-terminal moiety of the protein exposed to the periplasm and probably involved in binding of porin precursors (39). Similarly, a report that a protein of the Tox75 subfamily has its N-terminal domain involved in substrate recognition and complex assembly and its C-terminal domain in pore formation is also consistent with our new model (32).

Unlike Omp85, Tox75 and Sam50/Tob55, which are all part of large hetero-oligomeric complexes, TpsB proteins are thought to function in isolation (12, 40). Our current data strongly argue that monomeric FhaC forms the functional transport unit. In contrast, another TpsB transporter, Haemophilus influenzae HMW1B, was shown to form homotetramers, similar to Tox75 (11, 41). In both cases, however, it was proposed that each monomer forms an independent translocation pore rather than the tetramer forming a central channel with a lipid-facing hydrophobic lining. If FhaC functions as a monomer, this implies that FHA is most likely not native for translocation through such a rather narrow channel (28). The degree of folding of the transported proteins during translocation is under debate also in the other Omp85/TpsB protein transport systems (12, 19, 20, 42, 43).

REFERENCES

1. Alder, N. N., and Theg, S. M. (2003) Trends Biochem. Sci. 28, 442–451
2. Matouschek, A., and Glick, B. S. (2001) Nat. Struct. Biol. 8, 284–286
3. Herrmann, J. M. (2003) Trends Microbiol. 11, 74–79
4. Reumann, S., and Keegstra, K. (1999) Trends Plant Sci. 4, 302–307
5. Thanssni, D. G., and Hultgren, S. J. (2000) Curr. Opin. Cell Biol. 12, 420–430
6. Schnell, D. J., Kessler, F., and Blobel, G. (1994) Science 266, 1007–1012
7. Wimley, W. C. (2003) Curr. Opin. Struct. Biol. 13, 404–411
8. Moslavac, S., Mirus, O., Bredemeier, R., Soll, J., von Haeseler, A., and Schleiff, E. (2005) FEBS Lett. 572, 1367–1371
9. Yen, M.-R., Peabody, C. R., Partovi, S. M., Zhai, Y., Tseng, Y.-H., and Saier, M. H. (2002) Biochim. Biophys. Acta 1562, 6–31
10. Jacob-Dubuisson, F., Fernandez, R., and Coutte, L. (2004) Biochim. Biophys. Acta 1699, 235–257
11. Schleiff, E., Soll, J., Kochler, M., Kuhlbrandt, W., and Harrer, R. (2003) J. Cell Biol. 160, 541–551
12. Paschen, S. A., Waizenegger, T., Stan, T., Preuss, M., Cytklaff, M., Hell, K., Rapaport, D., and Neupert, W. (2003) Nature 426, 862–866
13. Kozjak, V., Wiedemann, N., Milenkov, D., Lohaus, C., Meyer, H. E., Guiraud, B., Meisanger, C., and Pfanner, N. (2003) J. Biol. Chem. 278, 48520–48523
14. Vouhouox, R., Bos, M. P., Geursten, J., Mols, T., and Thomas, J. M. (2003) Science 299, 262–265
15. Gentle, J., Gabriel, K., Beech, P., Waller, R., and Litgby, T. (2004) J. Cell Biol. 164, 19–24
16. Bolte, B., Soll, J., Schulz, A., Hinnah, S., and Wagner, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15831–15836
17. Eckart, K., Eichacker, L., Sohrt, K., Schleiff, E., Heins, L., and Soll, J. (2002) EMBO Rep. 3, 557–562
18. Körninger, U. W., Hobbie, S., Benz, R., and Braun, V. (1999) Mol. Microbiol. 32, 1212–1225
19. Hinnah, S. C., Hill, K., Wagner, R., Schlüchter, T., and Soll, J. (1997) EMBO J. 16, 7351–7360
20. Hinnah, S. C., Wagner, R., Sveshnikova, N., Harrer, R., and Soll, J. (2002) Biochips J. 83, 899–911
21. Jacob-Dubuisson, F., El-Harel, C., Saint, N., Guedin, S., Willery, E., Molé, G., and Locht, C. (1999) J. Biol. Chem. 274, 37731–37735
22. Sveshnikova, N., Grimm, R., Soll, J., and Schleiff, E. (2000) Biol. Chem. 381, 687–693
23. St. Gemen, J. W., III, and Grass, S. (1998) Mol. Microbiol. 27, 617–630
24. Güdér, S., Willery, E., Tammasson, J., Forte, E., Droebeg, H., Locht, C., and Jacob-Dubuisson, F. (2000) J. Biol. Chem. 275, 30202–30210
25. Jacob-Dubuisson, F., Locht, C., and Antoine, R. (2001) Mol. Microbiol. 40, 306–313
26. Philipp, A., Phale, P. S., van Gelder, P., Rosenbusch, J. P., and Koebnik, R. (1998) FEBS Lett. 420, 65–72
27. Jacob-Dubuisson, F., Ruissen, C., Mielcarek, N., Clement, E., Menozzi, F. D., and Locht, C. (1996) Mol. Microbiol. 19, 65–78
28. Güdér, S., Willery, E., Locht, C., and Jacob-Dubuisson, F. (1998) Mol. Microbiol. 29, 763–774
29. Coutte, J., Antoine, R., Droebeg, H., Locht, C., and Jacob-Dubuisson, F. (2001) EMBO J. 20, 5040–5048
30. Andersen, C., Bachmeyer, C., Tauber, H., Benz, R., Wang, J., Michiel, V., Newton, S. M., Hofnung, M., and Charbit, A. (1999) Mol. Microbiol. 32, 851–867
31. Andersen, C., Hughes, C., and Koronakis, V. (2002) J. Membr. Biol. 185, 83–92
32. Ertel, F., Mirus, O., Bredemeier, R., Moslavac, S., Becker, T., and Schleiff, E. (2005) J. Biol. Chem. 280, 28281–28289

7 H. Hodak and F. Jacob-Dubuisson, unpublished data.
33. Surrey, T., and Jahnig, F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7457–7461
34. Surrey, T., Schmid, A., and Jahnig, F. (1996) Biochemistry 35, 2283–2288
35. Danelon, C., Brando, T., and Winterhalter, M. (2003) J. Biol. Chem. 278, 35542–35551
36. Yang, F. L., and Braun, V. (2000) Int. J. Med. Microbiol. 290, 529–538
37. Sanchez-Pulido, L., Devos, D., Genevrois, S., Vicente, M., and Valencia, A. (2003) Trends Biochem. Sci. 28, 523–526
38. Reumann, S., Davila-Aposte, J., and Keegstra, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 784–789
39. Voulhoux, R., and Tommassen, J. (2004) Res. Microbiol. 155, 129–135
40. Wu, T., Malinverni, J., Ruiz, N., Kim, S., Silhavy, T. J., and Kahne, D. (2005) Cell 121, 235–245
41. Surana, N. K., Grass, S., Hardy, G. G., Li, H., Thanassi, D. G., and Gme, J. W., III (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 14497–14502
42. Clark, S. A., and Theg, S. M. (1997) Mol. Biol. Cell 8, 923–934
43. Johnson, A. E., and Jensen, R. E. (2004) Nat. Struct. Mol. Biol. 11, 113–114