The chaperone/usher system is one of the best characterized pathways for protein secretion and assembly of cell surface appendages in Gram-negative bacteria. In particular, this pathway is used for biogenesis of the P pilus, a key virulence factor used by uropathogenic Escherichia coli to adhere to the host urinary tract. The P pilus individual subunits bound to the periplasmic chaperone PapD are delivered to the outer membrane PapC usher, which serves as an assembly platform for subunit incorporation into the pilus and secretion of the pilus fiber to the cell surface. PapC forms a dimeric, twin pore complex, with each monomer composed of a 24-stranded transmembrane β-barrel channel, an internal plug domain that occludes the channel, and globular N- and C-terminal domains that are located in the periplasm. Here we have used planar lipid bilayer electrophysiology to characterize the pore properties of wild type PapC and domain deletion mutants for the first time. The wild type pore is closed most of the time but displays frequent short-lived transitions to various open states. In comparison, PapC mutants containing deletions of the plug domain, an α-helix that caps the plug domain, or the N- and C-terminal domains form channels with higher open probability but still exhibiting dynamic behavior. Removal of the plug domain results in a channel with extremely large conductance. These observations suggest that the plug gates the usher channel closed and that the periplasmic domains and α-helix function to modulate the gating activity of the PapC twin pore.

The cell envelope of Gram-negative bacteria contains a vast array of protein machineries dedicated to the translocation of polypeptides across the cytoplasmic membrane, periplasm, and outer membrane (OM)1 (1, 2). Some of these complexes also participate in the assembly of surface-exposed appendages, such as flagella and pili (fimbriae). One of the most thoroughly studied secretion systems is the chaperone/usher pathway, responsible for the biogenesis of a superfamily of virulence-associated surface structures, including P and type 1 pili (3). These pili play essential roles in the pathogenesis of uropathogenic E. coli by providing a tool for attachment of the bacteria to host urothelial cells (4–6). P pili, encoded by the chromosomal pap gene cluster, are critical virulence factors for infection of the kidney by uropathogenic E. coli and the development of pyelonephritis. The P pilus is composed of multiple subunits of PapA, which form a rigid helical rod. A thin linear tip fibrillum is located at the distal end of the pilus and is made of four different subunits (PapK, PapF, PapE, and the adhesin PapG) that assemble in a precise order and stoichiometry (3). The minor pilin, PapH, anchors the pilus rod to the cell surface (7).

The P pilus subunits are first translocated through the cytoplasmic membrane via the Sec general secretory pathway (8). Once in the periplasm, the subunits form binary complexes with the PapD chaperone. The details of the binding interaction between the chaperone and subunits were revealed by crystal structures of chaperone-subunit complexes (9–11). The actual assembly of the subunits into a pilus and secretion of the pilus fiber to the cell surface is mediated by the OM usher, PapC (12). The usher recruits chaperone-subunit complexes from the periplasm and provides a platform for polymerization of the subunits in a precise order (13, 14). The energy for pilus formation at the OM is thought to be provided by the polymerization itself, and the details of the interactions between subunits during the polymerization process are well understood (11, 15, 16). Nevertheless, how the usher facilitates polymerization and how fiber growth is coupled to translocation are two currently unresolved questions.

The PapC usher is a dimer where each monomer is composed of four domains: 1) a N-terminal periplasmic domain (~135 residues), 2) a β-barrel domain (residues 135–640), 3) a plug domain (residues 257–332) located within the β-barrel domain, and 4) a periplasmic C-terminal domain (residues 641–809) (17–19). The N-terminal domain of the usher has been implicated in the recognition and initial binding of chaperone-subunit complexes (20, 21). The C-terminal domain participates in the binding of chaperone-subunit complexes and is required for further assembly (17, 22). A major breakthrough in our understanding of the chaperone/usher system came with the elucidation of the three-dimensional structure of the usher translocation channel (19). The crystal structure of a 55-kDa fragment corresponding to the predicted transmembrane domain (residues 130–640) revealed that the pore is a kidney-shaped β-barrel of 24 strands (see Fig. 1A, blue), the largest number of strands identified for OM proteins so far. The inner dimensions of the pore are extremely large (25 × 45 Å), and it is likely that a gaping pore of such dimensions would be deleteri-
ous to the cell. Thus, not surprisingly, the pore is occupied by a plug domain, formed by the region between strands β6 and β7, which adopts a six-stranded β sandwich fold (see Fig. 1A, orange). The plug domain appears to be held in place by a hairpin loop between strands β5 and β6 (see Fig. 1A, magenta). Finally, another salient feature of the PapC pore is the presence of an α-helix (residues 448–465) that interacts with the β5–6 hairpin and is positioned over the hairpin and plug domain on the extracellular side of the barrel lumen (see Fig. 1A, aqua).

There is evidence that PapC indeed functions as a dimer during pilus biogenesis. PapC was shown to assemble as a dimeric twin pore complex in the lipid bilayer by cryo-EM (23) and in solution by crystallography (19). In addition, complementation of defective PapC mutants was achieved by expression of the homologous FimD usher used in type 1 pilus biogenesis (17, 22), and PapC-FimD interactions were demonstrated (22). Recently, a type 1 pilus intermediate was isolated, and a snapshot of the pilus biogenesis process was captured by cryo-EM of this complex (19). The images revealed electron densities for the FimD usher that corresponded to the crystal structure of the PapC dimer. In addition, the images revealed that the assembling pilus fiber appears to utilize only one of the usher monomers for secretion. Based on the structural and biochemical information obtained so far on both the P pilus and the type 1 pilus systems, a model for pilus assembly at the usher was presented (19) whereby the periplasmic N-terminal domains of both usher monomers alternate in delivering the subunits to an assembly platform provided by the usher dimer, whereas translocation through the OM occurs via a single pore.

To gain insight into the molecular mechanism of the usher during pilus assembly and secretion, we have initiated an electrophysiological analysis of the pore function of PapC. Here we used the planar lipid bilayer technique to investigate the channel properties of the PapC usher and some of its mutants. The comparison of the functional properties of the wild type and mutant proteins allowed us to gain insight into the role of various domains in the basal activity of the usher channel and set the stage for using electrophysiology as a functional assay for secretion.

**EXPERIMENTAL PROCEDURES**

**Media and Reagents**—Dodecyl-maltopyranoside and lauryl(dimethyl)amine oxide were purchased from Anatrace, and N-octyl-oligo-oxethylenc was from Axxora. Thrombin was from Novagen. The pentane and hexadecane used in planar lipid bilayer experiments were from Burdick and Jackson and TCI, respectively. Other chemicals were from Sigma or Fisher.

**Strains, Plasmids, and Growth Conditions**—E. coli strains DH5α (24) and the multi-porin mutant BL21(DE3)omp8 (25) were used for plasmid construction and PapC purification, respectively. Bacteria were grown in LB broth containing 100 μg/ml ampicillin at 37 °C with agitation. For protein expression, PapC was induced at an A600 of 6.0 for 2–2.5 h by the addition of 0.1% L-arabinose. Plasmid pDG2 encoding wild type PapC with a thrombin cleavage site and a His6 tag added to the C terminus was previously described (23). New plasmids were constructed from pDG2 to engineer the various mutations. A detailed description of the strategies and the primer sequences are given in supplemental Fig. S1. All of the PapC mutants were checked for proper construction by DNA sequencing.

**PapC Purification**—The OM fraction from bacteria induced for expression of one of the PapC constructs was isolated by French press disruption and Sarkosyl extraction, as described (22). The OM was solubilized by resuspending into 20 mM Tris-HCl (pH 8), 0.12 M NaCl, and 1% dodecyl-maltopyranoside and rocking overnight at 4 °C. The extract was clarified by ultracentrifugation (100,000 × g, 1 h, 4 °C), imidazole was added to 20 mM to the supernatant fraction, and this fraction was run over a nickel affinity column (HisTrap; GE Healthcare) using an Akta fast protein liquid chromatography apparatus (GE Healthcare). The bound PapC protein was eluted using an imidazole step gradient in buffer A, which is 20 mM Tris-HCl (pH 8), 0.12 M NaCl, and 10 mM lauryl(dimethyl)amine oxide. Fractions containing PapC were pooled, and the His6 tag was cleaved off PapC by overnight digestion at room temperature with 1.5 units of thrombin/mg of PapC while dialyzing against 20 mM Tris-HCl (pH 8) and 0.12 M NaCl. The thrombin was inhibited by addition of phenylmethlysulfonyl fluoride, and the mixture was subjected to a second round of nickel affinity chromatography in buffer A. In this case, the thrombin-cleaved PapC came off the column in the flow-through fraction. The collected flow-through material was concentrated using a Millipore Ultrafree centrifugal concentrator (molecular mass cut-off, 50 kDa) and applied to a HiLoad 16/60 Superdex 200 prep grade gel filtration column (GE Healthcare) in buffer A. The peak fraction containing PapC was collected from the gel filtration column and concentrated using the centrifugal concentrator as described above. Purified protein was stored frozen in aliquots.

**Analysis of PapC Mutants for Expression and Function in Pilus Biogenesis**—Each of the PapC mutant constructs was compared with the wild type PapC for expression level in the OM by immunoblotting with anti-His6 tag (Convance) or anti-PapC antibodies. Proper folding of the PapC constructs in the OM was checked by resistance to denaturation by SDS, as determined by heat-modifiable mobility on SDS-PAGE. These procedures were performed as described (20, 22). The ability of each of the PapC mutants to complement a ΔpapC pap operon for assembly of P pili was determined by hemagglutination assay and purification of pili from the bacterial surface as described (22).

The release of pilus subunits into culture supernatant fractions was analyzed as follows. Bacteria expressing WT PapC or one of the PapC mutants together with a ΔpapC pap operon as described above were grown to mid-log phase and placed on ice. Five ml of each culture was centrifuged (3,000 × g for 10 min at 4 °C) to separate the bacteria from the culture supernatant. The supernatant was removed to a new tube, centrifuged again (10,000 × g for 10 min at 4 °C) and passed through a 0.22-μm filter (Millipore) to remove any remaining bacteria. The bacterial pellet was resuspended in 1 ml of 10 mM Tris (8.0), and an aliquot of the resuspended bacteria was mixed with an equal volume of 2× SDS-PAGE sample buffer and heated at 95 °C for 10 min. The cell-free culture supernatant was precipitated by the addition of 0.5 ml of 100% trichloroacetic acid and incubation on ice for 30 min. The precipitate was pelleted (10,000 × g for 20 min at 4 °C), washed twice with acetone,
resuspended in 100 μl of \( 1 \times \) SDS-PAGE sample buffer, and heated at 95 °C for 10 min. Twelve μl of the whole bacterial sample (representing 0.06 ml of the original culture) and 5 μl of the trichloroacetic acid-precipitated culture supernatant (representing 0.5 ml of the original culture) were subjected to SDS-PAGE, and chaperone-subunit complexes were detected by immunoblotting with anti-PapDG or anti-PapDE antibodies.

**Planar Bilayer Electrophysiology**—A 100-μm-diameter hole was formed in a 0.01-mm-thick Teflon film and pretreated with a 1% solution of hexadecane in pentane. The Teflon film was sandwiched between two Teflon chambers filled with 1.5 ml of 1 m KCl, 5 mM Hepes (pH 7.2). Fifty μg of soybean phospholipids (phosphatidylcholine Type II-S; Sigma) in pentane (5 mg/ml) were added to both chambers. A lipid bilayer was formed over the hole by lowering and raising the level of buffer in one chamber. An aliquot of pure protein was added to one chamber only (cis-side), from a 5–100-fold dilution of the purified protein (0.3–0.6 mg/ml) in a 1% N-octyl-oligo-oxyethylene solution in the 1 m KCl buffer. If no spontaneous insertion of channels occurred within 10 min, the membrane was broken and formed again, and/or more protein was added. The amount of protein added varied considerably depending on the success of insertion (10–7,000 ng added to the chamber).

Currents were recorded under voltage-clamp conditions with an Axopatch 1D amplifier connected to a CV-4B headstage (Axon Instruments). The voltages given are those of the cis-compartment with respect to the trans compartment (ground). The currents were filtered at 1 kHz and digitized every 100 μs (Instrutech). The data were stored on a PC computer using the Acquire software (Bruxton) and analyzed with pClamp.

**RESULTS**

**Phenotypic Characterization of the PapC Mutants**—We constructed a series of mutations in PapC to investigate the roles of major domains and features in pilus biogenesis and channel function. The following regions were deleted either alone or in combination (Fig. 1B): the N- and C-terminal domains (ΔNΔC), the plug domain (Δplug), the β5–6 hairpin that interacts with the plug (Δhairpin), and the α-helix that caps the hairpin (Δhelix). The PapC ΔNΔC mutant corresponds to the usher translocation domain for which the structure was recently determined (Fig. 1A) (19). Each of the PapC mutants was compared with the wild type protein for expression level in one chamber only (cis-side), from a 5–100-fold dilution of the purified protein (0.3–0.6 mg/ml) in a 1% N-octyl-oligo-oxyethylene solution in the 1 m KCl buffer. If no spontaneous insertion of channels occurred within 10 min, the membrane was broken and formed again, and/or more protein was added. The amount of protein added varied considerably depending on the success of insertion (10–7,000 ng added to the chamber).

Functionality of the PapC deletion mutants was assessed by the ability to complement a ΔpapC pap operon for assembly of pili on the bacterial surface and agglutination of red blood cells (Table 1). As expected based on previous studies (20–22), deletion of the N- and C-terminal domains abolished the ability of the usher to assemble pili. The Δplug mutant was also unable to assemble pili, demonstrating an essential functional role for this domain. In contrast, the mutation affecting the β5–6 hairpin (Δhairpin) had only mild effects on pilus biogenesis, and the Δhelix mutant behaved essentially as WT (Table 1). However, the combined ΔhelixΔhairpin mutant was completely defective for assembly on the bacterial surface. The ability of the Δhairpin mutant to assemble pili was surprising giving its low expression.
level (Table 1). This mutant may be stabilized by interactions with chaperone-subunit complexes and the assembling pilus fiber. Although the ΔNΔC, Δplug, and ΔhelixΔhairpin PapC mutants were unable to assemble pili on the bacterial surface, these mutants, particularly Δplug, might allow release of unassembled pilus subunits into the extracellular milieu. However, comparison of culture supernatant fractions from bacteria expressing WT PapC or one of the deletion mutants together with a ΔpapC pap operon did not show increased release of pilus subunits (data not shown).

Channel Signatures of Wild Type and Mutant PapC—The channel properties of purified wild type and mutant PapC proteins were investigated with the planar lipid bilayer technique. The Δhairpin, ΔhairpinΔhelix, and the ΔNΔCΔplug mutants were not analyzed for channel formation because of their effects on protein stability and/or expression. In the planar lipid bilayer technique, a phospholipid bilayer is formed over a 100-μm-diameter hole in a Teflon film separating two buffer-filled chambers, and an aliquot of purified protein in detergent is added to one chamber only (the cis-side). Gentle stirring of the chamber promotes the spontaneous insertion of single pore-forming protein. Under voltage-clamp conditions, such insertion events are typically witnessed by a change in the amount of current passing through the bilayer and a change in the pattern of current fluctuations. Some proteins are more prone to rapid insertion; the E. coli porin OmpF, for example, inserts within a few minutes. We found that the wild type and mutant PapC proteins were relatively reluctant to insertion. An allowance of up to 10 min was set for insertions, and often several attempts were necessary, even with amounts 7,000 times larger than those typically used for OmpF porin (~1 ng). We attribute this intrinsic reluctance to the complex structural organization of the usher protein, with two subunits comprising a 24-stranded β-barrel domain and large N- and C-terminal domains. Despite this technical difficulty, we were able to obtain multiple recordings of each protein investigated here. We have not yet been able to experimentally determine the orientation of the inserted proteins, but we assume that the protein inserts with the extracellular side going in first, because it is unlikely that the hydrophilic N- and C-terminal domains would be able to go through the bilayer.

Fig. 2 shows typical recordings of the spontaneous activity of the wild type PapC and the ΔNΔC, Δplug, and Δhelix mutants. The recordings were made in buffer containing 1 mM KCl, which is a typical composition for the initial characterization of pore-forming proteins when using this technique, because it stabilizes the membrane and provides a good signal-to-noise ratio because of the large currents at high ionic concentration. The four protein types have very distinct kinetic signatures, which were found repeatedly in different experiments (>7 for wild type PapC, 5 for ΔNΔC, 9 for Δplug, and 8 for Δhelix). The fact that each protein has a characteristic activity pattern underscores that the observed activity is not artifactual and does not originate from the instability of the membrane. All four channels show spontaneous opening and closing transitions that are seen as spiky deflections of the current traces. These recordings were made at a clamped potential of −30 mV, and by convention openings are shown as downward spikes (marked by the downward arrows in Fig. 2, A and B). It is noteworthy that, in the same conditions, the Δplug channel passes a huge amount of current, with a maximum current level of several hundreds of pA at this relatively low voltage (for comparison, the current passing through the three channels of a trimeric OmpF porin is about 120 pA at 30 mV) (27). In fact, we found that we need to maintain the membrane potential below 50 mV and limit the recordings to no more than 30 s to avoid difficulties in clamping the membrane potential. The Δhelix channel shows bursts of activity comparable with that of the Δplug channel, although the size of the events is smaller. Another remarkable trait is that none of these channels is either completely closed or completely open, as might have been expected for the wild type and Δplug PapC, respectively, and all show a substantial amount of dynamic behavior. In the text below, we describe the activities in further detail.

Activity of Wild Type PapC—The trace in Fig. 2A shows that the current through the wild type channel remains at a baseline level that is close to the zero current level but displays frequent short-lived transitions to open states of various sizes. In Fig. 3, we display the traces on an expanded time scale to show the details of the opening activity. All four traces in Fig. 3 were obtained from the same bilayer at +50 mV (A and B) and −50 mV (E and F). C and D of Fig. 3 show amplitude histograms constructed from 1-min-long recordings in the same experiment at +50 and −50 mV, respectively. Such histograms represent the number of sample points having the current values given in the abscissa (bins are 0.5 pA). The peaks correspond to

| Plasmid | HA titer | Pilus assembly | Expression | SDS resistance |
|---------|----------|---------------|------------|---------------|
| pDG2    | 128      | WT            | WT         | WT            |
| ΔNΔC    | pNH270   | None          | WT         | Reduced       |
| Δplug   | pNH281   | None          | Reduced    | Reduced       |
| ΔNΔCΔplug | pNH294  | ND            | Low        | None          |
| Δhelix  | pNH269   | WT            | Reduced    | WT            |
| Δhairpin | pNH268   | Low           | WT         | WT            |
| ΔhelixΔhairpin | pNH271 | None         | Low        | WT            |

*HA (hemagglutination assay) titer is the fold dilution of bacteria that can still agglutinate human red blood cells.

**Pilus assembly is amount of pili assembled on the bacterial surface, as measured by pilus purification.

**Expression is the expression level of the PapC construct in the OM.

**SDS resistance is resistance of OM-localized PapC to denaturation by SDS, as determined by heat-modifiable mobility on SDS-PAGE.

**WT, similar to wild type PapC.

**Reduced, moderately lower compared with wild type PapC.

**ND, not determined.

**Low, very low compared with wild type PapC.
Channel Properties of the PapC Usher

A. Wildtype

B. ΔNΔC

C. Δplug

D. Δhelix

FIGURE 2. Kinetic signatures of the wild type and mutant PapC channels. Representative 20-s traces of single channels were obtained in 1 M KCl at a membrane potential of −30 mV. Tick marks indicate the current levels for the C state; downward deflections are channel openings, as indicated by the downward arrow. The Δplug channel spends most of the time in a highly open state (marked O2); a smaller open current level is also observed (marked O1). These two states are also clearly defined in the trace of the Δhelix channel. The dashed lines are the zero current level. Except for the ΔNΔC mutant, the wild type and the other two mutant channels show a leak between the current level of the annotated closed state (C) and the zero current level (22 pA for WT, 57 pA for Δplug, and 19 pA for Δhelix, in these particular experiments). Note that all traces are shown at the same scale.

the most frequently observed current values. For example in C, the histogram displays two major peaks at ~70 and 100 pA. The 70-pA peak corresponds to the level marked BL (base line) in A and B. The peak labeled I indicates that transitions to this current level are frequent and/or prolonged. Visual inspection of traces A and B indeed shows that the channel frequently opens to a level marked by the dotted line (1). These openings are well defined and have a clear-cut square top, which is the canonical signature for bona fide transitions during channel gating. The size of these openings is therefore about 30 pA, the difference between the levels BL and I. Similarly, the recording at −50 mV shows a base-line peak of approximately −35 pA with additional peaks at approximately −60 pA (peak 1) and −90 pA (peak 2). The transitions to these levels are apparent in traces E and F.

However, these traces clearly illustrate that well defined openings to other current levels also occur. Remarkably, the size of other openings are varied and do not appear to be integer-multiples of 30 pA. Two of these, of 190 and 105 pA, are highlighted in traces A and B, respectively, and one at 146 pA is shown in F (numbers in angled brackets represent the current difference from the base line). These transitions are not frequent or long-lived enough to yield peaks on the amplitude histograms. The diversity of current levels has made it impossible to generate current-voltage relationships, because we do not know what current steps belong to the same type of events while the voltage is being changed. There also does not seem to be strong reproducibility in the size and the frequency of these events from one bilayer to the next.

Finally, we have seen some variability in the current value for the base line. A current value of 70 pA, such as seen in Fig. 3 (A and B), is rather high for a true closed state. There is indeed a leak through the bilayer (or at the bilayer-Teflon interface), but such a leak is typically on the order of a few pA. Close inspection of many traces shows that, in fact, the base-line level might represent a very stable, already partially open state of the channel, because a closing step is observed from this level on rare occasions. This phenomenon is also observed in the Δhelix and the Δplug mutants (Fig. 2).

In summary, the pattern of activity of the wild type channel is reproducible and characterized by the following traits: 1) the channel is mostly in a leaky, low conductive state, 2) the channel shows additional transient openings to many different current levels that are not integer multiples of each other, 3) some of the current levels can be quite large, 4) transitions to the various current levels typically involve a single step, with no dwelling at intermediate levels. This kinetic signature suggests that the bilayer contains a single protein, although we do not have direct proof that this is the case. However, if multiple proteins were present, the larger events would be composites of smaller ones with a transient step, because it would be unlikely that several proteins gate in perfect unison. We also have to consider that the protein is dimeric and thus can expect to occasionally see two events of the same size stacked on one another, as seen in Fig. 3F. But again, there is no direct proof at this point that these stacked events originate from two different monomers rather than one.

The ΔNΔC Mutant Is a Mostly Open Channel—The N- and C-terminal domains have been shown to play essential roles in pilus assembly (17, 20–22). The structure of the N-terminal
Channel Properties of the PapC Usher

The channel properties of the PapC usher were determined and shown to form a soluble, globular domain (21). The structure of the usher C-terminal domain is unknown but is believed to form a similar globular domain. We were interested in testing whether these domains might modulate the basal channel activity of PapC by analyzing a mutant deleted in both domains. The truncated protein was even more reluctant to insert than wild type PapC, even if reconstituted first in artificial liposomes and then added to the bilayer. A reproducible activity was found in five record-

ings, one of which contained what appears to be a single dimer and is shown in Fig. 4 (A–C). The traces reveal a channel that is essentially open most of the time (\(P_o\), the probability to be in the open state, is 0.9) and undergoes transient closures to two well defined current levels. Marked segments are shown on an expanded time scale below the main traces. In principle, there could be three possible scenarios to account for these data: 1) the activity originates from two monomers within one dimer, 2) it originates from two monomers belonging to two distinct dimers, where only one monomer from each is active, and 3) it originates from two separate entities that inserted as monomers. We can dismiss the third possibility on the basis that the proteins were purified as dimers (supplemental Fig. S2) and should insert as such. Of the other two possibilities, we believe that the first one is most likely, because of the high level of cooperativity observed in the opening and closing transitions (Fig. 4, see expanded traces in A and B and also see D). Therefore, the simplest explanation for the observed activity is that a single dimer was reconstituted in the experiment shown in Fig. 4 (A and B), and we are witnessing the activity of each monomer, either individually or cooperatively.

For this reason, we are assigning current levels to the closed (C), open monomer (O1), and open dimer (O2) states as indicated. In this experiment, transitions to the O1 level were not frequent enough to yield a peak in the amplitude histograms displayed to the right of the main traces. These histograms, however, do show that the major peak corresponds to the O2 level.

Fig. 4C is a current-voltage (I/V) plot generated from this experiment, where the size of the current difference between the closed level and each of the O1 and O2 levels has been plotted versus voltage. The slope of the linear regression gives the so-called channel conductance, which is indicative of the channel size. Conductance depends not only on the pore dimensions but also on the interactions between the permeant ions and the channel wall. Nevertheless, a large conductance is typically found in wide channels, such as porins (28) and other translocons (29, 30). The conductances derived from these plots are 799 and 1612 pS for the O1 and O2 pores, respectively. The fact that these two conductances differ by a factor of 2 lends support to the notion that they represent the monomeric and dimeric channels. The O1 conductance is in the range of the translocons (29, 30). The conductances derived from these plots are 799 and 1612 pS for the O1 and O2 pores, respectively.

![Dynamic activity of the wild type PapC channel](image)

**FIGURE 3. Dynamic activity of the wild type PapC channel.** Representative traces at +50 mV (A and B) and −50 mV (E and F) showing the details of the closed channel behavior of wild type PapC. The channel spends most of the time at a main base-line current level (labeled BL) from which upward (+50 mV) or downward (−50 mV) flickerings represent opening transitions. These transitions are frequent and to variable current levels. Amplitude histograms were constructed from 1-min-long recordings in the same experiment at +50 mV (C) and −50 mV (D). They show a major peak corresponding to the base line. But only one or two peaks (labeled 1 and 2) corresponding to the most prominent open levels are observed. The levels are highlighted by dotted lines in the traces. The numbers in angled brackets correspond to the size of selected larger transitions measured from the base line.
Channel Properties of the PapC Usher

**FIGURE 4.** The $\Delta$NAC channel is mostly open. A and B, Representative 5-s traces of a single dimer of the $\Delta$NAC mutant at $+50$ mV (A) and $-50$ mV (B). The channel is largely in an open state (marked by O2) but displays frequent short-lived downward ($+50$ mV) or upward ($-50$ mV) transitions representing closing events, either to the closed state (marked C) or a state of lower conductance (marked O1). The dashed lines represent the zero current level. The scale bar gives the values in the following order: regular trace/expanded trace. The amplitude histograms at the right side of the trace were obtained from 13 s of recording of the same traces. C, current-voltage relationships of the monomeric (O1) and dimeric current (O2). The conductance was calculated as the slope of each linear regression and was found to be 799 and 1,612 pS for O1 and O2, respectively. D, representative 1.8-s current trace obtained at $-50$ mV from a bilayer that contained more than one channel. To save space, the zero current level is not shown, but the maximum current observed is given ($-245$ pA). From this level, we can estimate that three open dimers are present in this bilayer. The trace shows the combined kinetics of the three dimers with well defined monomeric (M) and dimeric (D) transitions. The current levels marked by the dashed lines correspond to multiples of the presumed monomeric conductance (800 pS or 40 pA). as this one, were obtained in three other occasions and also displayed the same activity pattern.

The $\Delta$plug Mutant Has an Extremely High Conductance—A salient feature of the PapC structure is the occlusion of the $\beta$-barrel lumen by a large globular domain acting as a plug (Fig. 1A). The absence of the plug is expected to yield a wide channel delimited by the 24 $\beta$-strands. Electrophysiological analysis confirms that this is indeed the case. The traces in Fig. 5 (A and B) reveal a dynamic channel that dwells at three preferred current levels. Based on the same arguments as we made for the $\Delta$NAC mutant above, we believe that these levels correspond to a closed and two open states representing the monomeric and dimeric forms from the same usher dimer. Note that these traces were obtained at a transmembrane potential of only $\pm15$ mV. Potentials higher than $\sim40$ mV led to so much current and activity that the traces became difficult to analyze (as can be surmised from the trace of Fig. 2C). The I/V plot of Fig. 5C was obtained from the same experiment shown in Fig. 5 (A and B). The linear regression through each plot provided conductance values of 2,950 pS for the O1 level, and 7,330 pS for the O2 level. As a reference, the conductance of a single monomer of the porin OmpF is 1,400 pS in the same conditions (27).

In terms of kinetics, we found that, surprisingly, the channel displays a lot of dynamic behavior and is not a gaping open pore, as might have been anticipated. It does appear to favor an open state, but numerous transitions to less conductive states are frequently observed, some of them even for prolonged periods of time (Fig. 5). An interpretation for this observed pattern is provided under “Discussion.” At potentials greater than 40–50 mV, the traces dwell at a very high current level, suggesting that the channel is mostly open, and the quality of the recording deteriorates somewhat because of the large amount of current and channel noise. In the experiment of Fig. 5, channel opening appears favored at negative potentials, as can be seen from the relative size of the peaks of the amplitude histograms, but this asymmetry has not been a consistent trend.

The $\Delta$helix Mutant Allows Occasional Plug Release—The single $\alpha$-helix in the PapC protein is located on the extracellular side, as a cap that covers the dent in the barrel wall because of the inwardly folding of the $\beta$5–6 hairpin (Fig. 1A). It is anticipated that removal of the helix might release the $\beta$5–6 hairpin keeping the plug domain in place. The electrophysiological recordings revealed that the $\Delta$helix mutant is somewhat destabilized and will occasionally burst into a gating pattern that is similar to the $\Delta$plug mutant. Because of the similarity in behavior with the $\Delta$plug mutant, our interpretation of these results is that, indeed, the plug domain can spontaneously dislodge for some period of time in this mutant. In some experiments, the $\Delta$plug-like behavior is apparent at the onset of the recording, as shown in Figs. 2D and 6 (A and B), as if the protein inserted in a conformation where plug movement had already occurred. Other times, the channel displays at first a quiet activity and then switches to a high activity mode. This switch may be preceded by a stretch of opening transitions to low conductance states (Fig. 6, C and D). This type of behavior has been observed in eight independent experiments, but there is variability in the time dependence, duration, and frequency of the $\Delta$plug-like activity mode. The conductance of the $\Delta$helix mutant is some-
DISCUSSION

We have presented here the first report on the electrophysiological activity of the wild type and mutants forms of the PapC

FIGURE 5. The Δplug channel has an extremely large conductance. Representative current traces of 20-s recording at +15 mV (A) and −15 mV (B). At positive voltages, the channel is largely in a closed state (marked C) but displays frequent opening transitions (upward) to O1 and O2 current levels. This kinetic behavior is reflected in the sizes of the peaks of the amplitude histogram shown at the right side of the trace. At negative voltages, however, the channel spends more time in the O2 state than in the closed state (openings shown as downward transitions). Highlighted segments are shown on an expanded time scale below each trace to illustrate the two current levels. Each trace represents 10 s, but the amplitude histograms shown on the right side were obtained from 30 s of the same recording. Note that the O1 peak of the amplitude histograms is either broad or biphasic because of two slightly different current levels for O1, depending on whether the state originates from the closed state or the O2 state. C and D, traces from two separate experiments showing that the Δplug-like behavior occurred with a time delay after applying a voltage of −50 mV and is preceded by gating to substates. For trace D, in fact, the burst of activity at the end of the trace still corresponds to a conductance that is about half of O1. The downward arrows at the right side of the traces point in the direction of channel opening. The leftmost scale bar is for traces A and B, and the rightmost scale bar is for traces C (400 pA ordinate) and D (200 pA) ordinate. The dashed line is the zero current level.

FIGURE 6. The Δhelix mutant displays bursts of Δplug-like activity. Current traces were obtained from three different channels to illustrate the range of activity. A and B, traces were obtained at +15 and −15 mV, respectively, from the same channel, which displayed Δplug-like activity from the onset of the experiment. The traces in the boxes correspond to expanded segments to show details of the gating to the O1 and O2 states. Each trace represents 10 s, but the amplitude histograms shown on the right side were obtained from 30 s of the same recording. Note that the O1 peak of the amplitude histograms is either broad or biphasic because of two slightly different current levels for O1, depending on whether the state originates from the closed state or the O2 state. C and D, traces from two separate experiments showing that the Δplug-like behavior occurred with a time delay after applying a voltage of −50 mV and is preceded by gating to substates. For trace D, in fact, the burst of activity at the end of the trace still corresponds to a conductance that is about half of O1. The downward arrows at the right side of the traces point in the direction of channel opening. The leftmost scale bar is for traces A and B, and the rightmost scale bar is for traces C (400 pA ordinate) and D (200 pA) ordinate. The dashed line is the zero current level.
Channel Properties of the PapC Usher

As found for other translocators involved in the biogenesis of membrane or surface structures, such as FhaC, Omp85, HMW1B, and PulD (29–32), the usher functions display characteristic size and kinetic signatures. The PapC mutants were also analyzed for effects on expression and folding of the usher in the OM and for function in pilus biogenesis. Removal of the plug domain and of the N- and C-terminal domains completely compromised pilus assembly on the bacterial surface. This was expected for the ΔNAC mutant, because the individual domains were previously shown to play roles in subunit recognition and binding (17, 20−22). Our results show that the plug domain is also essential for PapC function, suggesting that the plug may participate in fiber assembly and secretion rather than acting simply as a channel gate. A similar functional role was recently reported for the plug domain of the Cfa1A usher involved in F1 capsule biogenesis in *Yersinia pestis* (33). The PapC Δplug mutant did not allow release of unassembled pilus subunits into the extracellular medium, suggesting that the subunits remained bound to the chaperone in the periplasm. Further studies are required to determine the specific defect of subunits remained bound to the chaperone in the periplasm.

Further studies are required to determine the specific defect of the Δplug mutant in pilus biogenesis. Although the combined ΔhelixΔhairpin mutant was defective for pilus biogenesis, neither the β3−6 hairpin nor the α-helix alone was required for function of the usher. However, the channel data support a role for the helix in maintaining the plug in a closed state. The lack of effect of the Δhelix mutation on pilus biogenesis suggests that the occasional destabilization of the plug is not sufficient to impair the overall function of the machine.

The comparison of the activity patterns of the wild type and mutant channels allows us to propose roles for the different domains in the basal pore activity of the usher, i.e. in the absence of chaperone-subunit complexes. The cartoons in Fig. 1B illustrate the domain composition of the different proteins investigated here. One might have expected the wild type channel to be closed shut because of the plug. However, current fluctuations in the bilayer data indicate that transitions between “open” (ion-conductive) and “closed” (non-ion-conductive) states frequently occur. The transitions display variable sizes, possibly indicating multiple routes for ion movement. Some of these transitions have sizes that are comparable with those of the Δplug channel. A close inspection of the PapC crystal structure reveals the presence of small water-filled cavities at the interface between the plug domain and the β-barrel wall (circled in red in Fig. 1C). These intrinsic “channels” could be a possible passageway for ions, while the plug is lodged inside the lumen. We propose that the transitions of various sizes correspond to 1) the interruption of the “water channels” by either the jiggling of the plug or the N- and C-terminal globular domains blocking the ion flow at the periplasmic end and 2) the occasional displacement of the plug to lead to the very large transitions. We expect plug displacement to be quite infrequent, short-lived, and even partial, because of the extensive network of interactions between the plug domain, the channel wall, and the β3−6 hairpin that dips inside the pore. We had hoped to investigate a mutant lacking the β5−6 hairpin, but the expression level of this protein was very low. On the other hand, we investigated a mutant lacking the α-helix that caps the β5−6 hairpin and likely participates in maintaining the plug in place (19). The behavior of the Δhelix mutant is indicative of a destabilized plug, because the channel will spontaneously enter a state of high conductance, comparable with that of the Δplug mutant. These transitions often occur in bursts and appear to be favored by negative voltages. The application of a transmembrane voltage might lower the energy barrier for plug displacement in this already unstable mutant. Interestingly, the conductance of the Δhelix mutant is smaller than that of the Δplug mutant, suggesting that the plug may not fully come out of the pore. These data support a proposed model where gating of the PapC translocation channel involves rotation of the plug domain within the pore lumen rather than movement of the plug completely out of the channel (19).

The Δplug mutant has a very large conductance, in line with the measured size of 45 × 25 Å for the translocation channel per se (19). Conductance is not only a reflection of the physical size of a pore but also of the interactions made between the channel wall and the permeating ions. Thus, we will refrain from calculating an estimated size on the basis of the monomeric conductance of ∼3,000 pS in 1 M KCl. This size is much larger than those reported for other protein translocators in the same ionic conditions, such as FhaC (1,200 pS) (29), HMW1B (1,400 pS) (30), and Omp85 (500 pS) (32), which may be related to the biological function of the PapC pore in translocating folded polypeptides or may be due to the large number of β-strands in PapC. Interestingly, oligomeric secretins such as XcpQ from *Pseudomonas aeruginosa* (34) and YscC from *Yersinia enterocolitica* (35) form channels with very large conductances (∼3−10 nS), which are comparable with that of the Δplug PapC mutant. EM studies revealed that the monomers organize as ring-like structures forming a central pore (34, 35). These observations are consistent with the notion that a fairly large pore is required for the translocation of folded substrates by secretion systems, either as a central conduit within a single subunit (as in PapC) or as the center of a ring of monomers (as in YscC and XcpQ). However, another secretin, PulD, showed a behavior similar to WT PapC, i.e. it appeared to be essentially closed but displayed voltage-dependent openings of various relatively small sizes (∼200 pS in a 400/100 mM KCl gradient) (31). The fluctuations were interpreted by the authors to represent slight movements of some protein domains.

An intriguing aspect of the behavior of the Δplug mutant is that a gating activity (i.e. open-closed transitions) is still observable, indicating interruptions in the ion flow. It is possible that these transitions derive from mobility of some of the barrel loops or other structural elements of the β-barrel itself or from the collapse of the barrel in the absence of the support of the plug. However, we favor the hypothesis that the transitions represent transient blockages of ion movement by the globular N- and/or C-terminal domains, which might have enough dynamic flexibility to transiently occlude the plugless pore. A triple ΔNΔCΔplug mutant, which would address the role of the N- and C-terminal domains in occluding the pore, did not express well in bacteria and did not appear to fold properly in the OM. Thus, electrophysiological investigations could not be performed on this mutant.

The PapC ΔNΔC mutant also shows gating activity despite lacking the N- and C-terminal domains. Here the plug is in...
place, and thus the gating activity is intrinsic to the plugged β-barrel and might originate from the jiggling of the plug or some other loops or domains affecting the size of the small water channels. These movements might be normally somewhat restricted in the presence of the N- and C-terminal domains. The observed transitions might also stem from the reduced stability of this particular mutant (as suggested by its reduced resistance to SDS) (Table 1). Interestingly, the large transitions observed in the WT and Δhelix proteins are absent in the ΔNΔC mutant. If indeed these transitions correspond to movements of the plug to liberate a large translocation channel, their absence in the ΔNΔC mutant might signify that the globular domains play a role in allowing the plug to occasionally come out in the wild type usher to yield these large transitions. This hypothesis fits with the proposal that the usher C-terminal domain is located under the pore and makes contact with the β5–6 hairpin and the plug domain (19), and the observation of a weaker density in the PapC channel in the absence of the C-terminal domain, as observed by cryo-EM (23).

In conclusion, the domain analysis of the PapC mutants presented here supports that PapC forms a twin pore and reveals that 1) ion movement is observable in WT, possibly through water-filled cavities at the interface of the plug and the barrel, 2) there is considerable dynamic flexibility in the whole protein, 3) the plugless channel has an extremely large conductance, in accord with the estimated dimensions and the biological activity of translocating folded peptides, 4) the α-helix capping the β5–6 hairpin appears to stabilize the plug in place, and 5) the N- and C-terminal domains might transiently occlude the pore and play a role in plug displacement. The knowledge of this basal activity sets the stage for further experiments on PapC in the presence of translocating substrates.

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