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Refolded Outer Membrane Protein A of *Escherichia coli* Forms Ion Channels with Two Conductance States in Planar Lipid Bilayers*

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Outer membrane protein A (OmpA), a major structural protein of the outer membrane of *Escherichia coli*, consists of an N-terminal 8-stranded β-barrel transmembrane domain and a C-terminal periplasmic domain. OmpA has served as an excellent model for studying the mechanism of insertion, folding, and assembly of constitutive integral membrane proteins *in vivo* and *in vitro*. The function of OmpA is currently not well understood. Particularly, the question whether or not OmpA forms an ion channel and/or nonspecific pore for uncharged larger solutes, as some other porins do, has been controversial. We have incorporated detergent-purified OmpA into planar lipid bilayers and studied its permeability to ions by single channel conductance measurements. In 1 M KCl, OmpA formed small (50–80 pS) and large (260–320 pS) channels. These two conductance states were interconvertible, presumably corresponding to two different conformations of OmpA in the membrane. The smaller channels are associated with the N-terminal transmembrane domain, whereas both domains are required to form the larger channels. The two channel activities provide a new functional assay for the refolding *in vitro* of the two respective domains of OmpA. Wild-type and five single tryptophan mutants of urea-denatured OmpA are shown to refold into functional channels in lipid bilayers.

The outer membrane of Gram-negative bacteria serves as a molecular sieve, resisting the entry of noxious compounds, while at the same time allowing the uptake of essential nutrients. Molecules up to about 600 Da in size are taken up by a set of more or less substrate-specific porins and transporters. Outer membrane proteins were among the first integral membrane proteins for which crystal structures have been solved by x-ray diffraction. In contrast to most proteins of the inner membrane, which are of the helical bundle type, the common structural motif of the outer membrane proteins is the β-barrel, *i.e.* an antiparallel β-sheet that closes on itself. Porins with 16 (OmpF, OmpC, PhoE (phosphophorin)),[1] 18 (LamB), or 22 (FhuA, FepA) antiparallel β-strands have been described (1–4). The outer walls of their structures are generally hydrophobic (to match the lipid bilayer), and they have water-filled central pores of variable sizes through which substrates are taken up. Whereas the porins are usually trimeric, the iron-siderophore transporters, FhuA and FepA, are monomeric. The substrate specificity of these proteins arises from specific residues in the pore, special peptide segments (aromatic "greasy slide" in LamB), or entire protein domains (periplasmic "cork" in FhuA). When incorporated into black lipid membranes, porins exhibit single channel activities that strongly depend on the particular porin, the applied transmembrane potential, and the type and concentration of the electrolyte in the environment. For example, OmpF of *Escherichia coli* has a conductance of 0.8 nanosiemens in 1 M NaCl, whereas the major porin from *Rhodopseudomonas blastica*, which is of similar size, exhibits a conductance of 3.9 nanosiemens in 1 M KCl (5, 6)

OmpA is another major outer membrane protein of *E. coli*. Although the mass of OmpA is similar to that of many other porins, OmpA consists of two separate domains. The N-terminal domain is integrated into the membrane in the form of a small β-barrel of only eight antiparallel β-strands. In addition, a C-terminal globular domain of approximately 150 residues extends into the periplasm. Unlike the porins, OmpA most likely exists as a monomer in the outer membrane. The crystal structure of the N-terminal domain of OmpA has recently been solved by x-ray diffraction (7). Compared with the larger porins, the barrel of this "miniporin" is very tight, with mostly hydrophilic residues and a few aqueous cavities closely packed in the lumen of the barrel. Providing structural stability to the cell appears to be one of the main functions of OmpA. This is probably accomplished by linking through the C-terminal domain the outer membrane to the periplasmic peptidoglycan. Additionally, OmpA mediates bacterial conjugation and functions as a receptor for various bacteriophages.

OmpA has also been reported to form channels or pores in lipid bilayers, although this aspect of the protein is somewhat controversial. Saint *et al.* (8) measured single channel conductance events in "solvent-free" planar bilayers on the order of 180 pS at 100 mV and in 0.25 M KCl. Based on osmotic swelling experiments with reconstituted OmpA proteoliposomes, Sugawara and Nikaido (9, 10) concluded that OmpA forms a diffusion channel of about 10 Å in diameter. However, only 2–3% of all OmpA molecules were in the open conformation in their preparation, and vesicles containing open channels could be separated from closed channel vesicles by density gradient centrifugation. These experiments indicated that the open and closed forms represent two different, relatively stable conformations of OmpA. The crystal structure of the N-terminal domain of OmpA, which was obtained from crystals formed from an OmpA fragment that was solubilized in the detergent C_8E_4, indicated no obvious aqueous pore, as the water-filled cavities were not connected in that structure (7). Based on their

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[1] The abbreviations used are: Omp, outer membrane protein; DPhPC, diphytanoylphosphatidylcholine; pS, picosiemens; LamB, maltophorin; Fhu, ferric hydroxamate uptake receptor; Fep, ferric enterobactin receptor.

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structure, these authors therefore questioned the ability of OmpA to form ion- or solute-conducting pores.

OmpA has also served as an excellent model to study the folding and membrane insertion of a constitutive integral membrane protein (11–13). In these studies, OmpA was refolded from 8 M urea by rapid dilution into a solution containing preformed lipid vesicles (or detergent micelles). OmpA seems to be ideally suited for these studies partly because it is a relatively small, yet polytopic integral membrane protein and also because as a β-barrel membrane protein it has a sequence of alternating hydrophobic and hydrophilic residues. This property makes the membrane-spanning sequences on average less hydrophobic than those of helical bundle membrane proteins, which therefore cannot be completely solubilized and unfolded in urea. However, a good functional assay to monitor the refolding of OmpA has been lacking up until now.

Because the formation of ion channels by OmpA has not been universally accepted and to develop a practical assay for the refolding of OmpA, we re-investigated the channel activity of OmpA in a well defined reconstituted lipid bilayer system. We found that both native OmpA and OmpA that was refolded in the detergent C_8E_4 forms two types of ion-conducting channels in planar bilayer membranes. The more frequent smaller channels exhibited a conductance of 50–80 pS and the less frequent larger channels a conductance of 260–320 pS in 1 M KCl at a 100 mV membrane potential. The smaller but not the larger conductance state was also observed when the N-terminal transmembrane domain, *i.e.* a fragment comprising residues 1–176, was refolded and incorporated into planar lipid bilayers. Five single tryptophan mutants of OmpA exhibited small and large channel conductances similar to those of wild-type OmpA.

EXPERIMENTAL PROCEDURES

Proteins—OmpA and single tryptophan mutants of OmpA were expressed in the OmpA-deficient *E. coli* strain MC4100rh$^-$ as described previously (13). These proteins were purified from the outer membranes by urea extraction and ion-exchange chromatography of the unfolded proteins in 8 M urea using a Q-Sepharose Fast Flow column (11). The transmembrane domain of OmpA Trp-7, Trp-7-(1–176), was generated from the single tryptophan mutant Trp-7 by utilizing site-directed mutagenesis to convert the codon for proline 177 to a stop codon. Briefly, the OmpA gene was transferred from pET1102 (13) into pAlter-1 (Promega, Madison, WI). The mutagenic primer 5′-CAGGGC-GAAGCAGCTTAAGTAGTTGCTCCGGC-3′, which also contained a unique site for *Afl*II and the commercial Amp selection primer (Promega), were used to introduce a stop codon at position 177. The mutagenized gene was reintroduced into pET1102 for expression in MC4100rh$^-$. The correct sequence was verified by sequencing. Trp-7-(1–176) was purified using essentially the same protocol as for OmpA, except that a final gel filtration step on a Superdex-75 HR column (Amersham Pharmacia Biotech) in 20 mM potassium phosphate, pH 7.3, 50 mM NaCl, containing 8 M urea, was added to separate the transmembrane domain from residual impurities. A sample of wild-type OmpA that was purified in its native form was a kind gift of Dr. Hiroshi Nikaido (University of California, Berkeley). This protein was derived from an OmpF- and OmpC-deficient K12-derivative *E. coli* strain (HN705) and was purified from the outer membrane by detergent extraction and repeated gel filtration over a Sephacryl S-300 column in dodecylmaltoside (14, 15). Native OmpA was suspended in 10 mM Tris-Cl buffer, pH 7.5, containing 0.4 M NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 0.1% dodecylmaltoside. For single channel measurements, these samples were diluted into a 20 mM micellar solution of tetraoxyethylene mono-*n*-octylether (C_8E_4, Bachem, Philadelphia, PA) to a concentration of 80 μg/ml, *i.e.* the same as that used for the refolded proteins that were obtained by the urea purification method.

Refolding of OmpA into Detergent Micelles—Refolding of OmpA and its derivatives was carried out as described in more detail by Kleinschmidt *et al.* (16). Briefly, 5 μl of a 4 mg/ml solution of unfolded OmpA in 15 mM Tris-Cl, pH 8.5, containing 8 M urea was diluted 50-fold into a 20 mM solution of C_8E_4 in 2 mM sodium borate, pH 10.0, containing 0.4 mM EDTA. The mixture was incubated overnight at 40 °C to ensure complete refolding of the protein. To remove misfolded protein aggregates the samples were centrifuged at 14,000 rpm in a table-top centrifuge (Eppendorff, Rexdale, Ontario) for 15 min prior to the addition to the planar membranes.

SDS-Polyacrylamide Gel Electrophoresis Analysis—SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (17). For checking the purity of the proteins, samples were diluted (1:1, v/v) with treatment buffer (0.125 M Tris-Cl, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.2% bromphenol blue), boiled at 100 °C for 5 min, and run on 12% polyacrylamide gels for the full-length OmpA proteins or 14% polyacrylamide gels for the transmembrane fragment. For measuring protein refolding by the gel-shift assay, the refolded samples were either boiled or incubated at 40 °C for 5 min and run on a 10–15% gradient polyacrylamide gel. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

Planar Lipid Bilayer Experiments—Planar lipid bilayers were formed from a solution of 17 mg/ml diphytanoylphosphatidylcholine (DPhPC, Avanti Polar Lipids, Birmingham, AL) in *n*-decane (Aldrich) using the method of Mueller *et al.* (18) with some modifications (19). The lipid solution was painted on a 500-μm hole in a Teflon partition separating two 1.5-ml compartments, which were filled with KCl buffer (1 M KCl, 10 mM Tris, pH 7.1). The compartments were connected to the recording system through two chlorided silver electrodes, one of which (the front, *cis* side) was grounded, whereas the other (the rear, *trans* side) was connected to a custom designed trans-impedance amplifier. The painted DPhPC/*n*-decane bilayer membranes were tested for integrity by checking the reflectance optically and also by their resistance and capacitance. After the bilayers were formed, 5 μl of 80 μg/ml of OmpA in C_8E_4 micelles were added to the *cis* compartment and stirred. Conductance measurements were made after about 10 min of equilibration with a 100-mV potential applied to the *trans* compartment. The current across the bilayer was recorded on magnetic medium. For analysis, the signal was filtered at 100 Hz with an 8 pole low-pass filter and digitized at 1 KHz (LABMAN, G. Szabo and C. Q. Ye, University of Virginia). Single channel conductance events were analyzed using TRANSIT (Baylor School of Medicine, Houston, TX), and IGOR (Wavemetrics, Portland, OR) software packages. Single channel conductance events were identified automatically in most traces. In a few traces where baseline noise was unusually large, it was difficult to identify transitions reliably by automated analysis. In such cases conductance steps were analyzed interactively using IGOR. The interactive and automated procedures yielded the same results, in terms of conductance levels and single channel histograms in all traces that were analyzed by both methods. The data were averaged from three to seven independent recordings, which all lasted for several minutes for each protein and condition.

RESULTS

Native OmpA Forms Two Types of Ion Channels in Planar Bilayers—A typical trace of a single channel recording of OmpA that was purified in its native form from the outer membranes of an *E. coli* K12-derivative strain by detergent extraction is shown in Fig. 1. In this (and all subsequent) experiment(s) OmpA in C_8E_4 micelles was added to the *cis* compartment next to DPhPC/*n*-decane bilayers and equilibrated, and a 100-mV potential was applied to the *trans* compartment. Two types of unitary conductance increases, indicated by downward deflections, were immediately induced. When in control experiments the detergent C_8E_4 alone was added in an amount equivalent to four times of that used in the mixed detergent/OmpA micelles, no channel activity was observed for several minutes (trace labeled C_8E_4 in Fig. 1). Three different conductance states are evident in the recording of native OmpA. In state I, all channels are closed, exhibiting a baseline conductance. In state II, a single small channel is open, exhibiting a conductance of the order of 50 pS, and in state III, a large channel with a conductance of the order of 320 pS is open. The trace begins at time A in state II with a single small channel open. At B, a large channel opens (state III) and closes again at C (state II). At D, the small channel closes to the baseline conductance state I. Between D and E, a small channel opens and closes. At E, a large channel opens directly from the baseline conductance state (state I → state III) but closes only to the open state of small channel at F (state III → state II), which closes at G to the baseline (state II → state I). A small channel opens again at

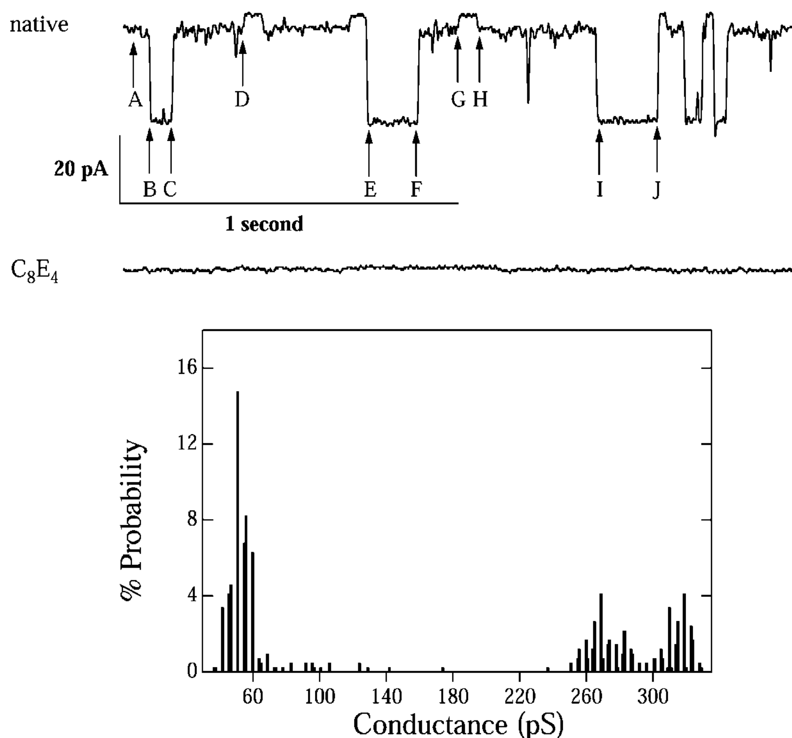

FIG. 1. **Single channel recording of native OmpA in planar lipid bilayers.** The bilayers were formed from DPhPC/*n*-decane. Both chambers were filled with 1 M KCl in 10 mM Tris-Cl buffer, pH 7.1; 0.2 μg/ml protein in C_8E_4 was added to the *cis* compartment, and a 100 mV potential was applied to the *trans* compartment. The trace is labeled to mark large and small conductance changes as described in the text. The trace of a control experiment with C_8E_4 (5 μl, 20 mM) in the absence of OmpA is also shown. The *lower panel* shows a histogram of the distribution of large and small channel openings and closings. A total of 413 events was analyzed.

H (state I → state II) and converts into a large channel at I (state II → state III), which closes directly to the baseline at J (state III → state I). A histogram of the single channel conductance levels of native OmpA is shown in the lower part of Fig. 1. The distribution of 413 total observed events shows three well separated peaks. Small channels of 40–60 pS conductance (mean 52 pS) accounted for 55% of all events. Two additional peaks corresponding to the state II ↔ state III (mean 268 pS) and state I ↔ state III (mean 317 pS) transitions are also evident.

One might ask whether the coexistence of small and large channels of OmpA reflects two separate populations of molecules that co-exist in planar bilayers or whether the observed channels originate from a single population with interchanging conformations. The following observations support the latter possibility: first, we never observed the simultaneous opening of two large channels or two small channels; and second, the large channels open and close either from/to the baseline or from/to the open state of a small channel, but small channels only open and close from/to the baseline and never from the open state of a large channel. Therefore, it appears that the small channels are kinetic precursors of the larger channels.

The Small and Large Channel Activities Are Regenerated in Refolded OmpA—OmpA is one of only a few proteins that can be refolded into preformed lipid bilayers. One goal of this study was to determine whether refolded OmpA exhibits the same single channel activity as native OmpA. A convenient assay to follow the refolding of OmpA is to monitor an increase in the mobility on polyacrylamide gels of the refolded relative to the unfolded proteins (20). This shift in mobility is only expressed in samples that are not boiled prior to electrophoresis. Upon denaturation by heat (or urea), OmpA runs on SDS gels as a single band that corresponds to an apparent molecular weight of 35 kDa, whereas native or refolded OmpA runs as a single band with an apparent molecular weight of 30 kDa (Fig. 2). Refolding of OmpA into lipid bilayers or detergent micelles can also be demonstrated by monitoring a change of the intrinsic Trp fluorescence (11, 16). When we incorporated refolded OmpA in C_8E_4 into planar lipid bilayers at the same concentrations as native OmpA, we again found unitary conductance values of similar magnitude as for the small and large channels of native OmpA. However, in contrast to the native protein, interconversions between the two conductance states were much rarer. In most cases, a single bilayer recording contained either small or large channels. About 80–85% of all recordings displayed predominantly small channels and about 15–20% predominantly large channels. Small and large channel recordings are displayed in Figs. 3 and 5, respectively. Because the data of the two types of channels are from different recordings (but still from the same refolded OmpA stocks), separate histograms are shown for the small and large channels in Figs. 4 and 5, respectively. There are precedents for the observation that single channels occur in two different but well defined

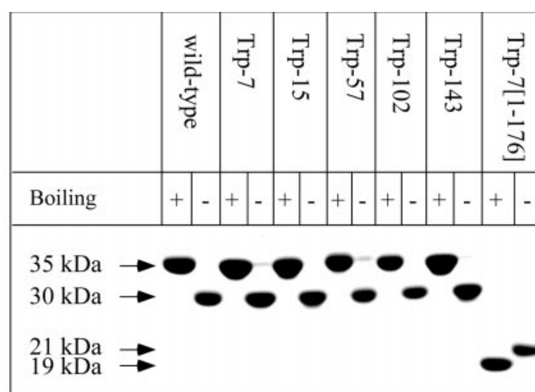

FIG. 2. **SDS-polyacrylamide gel electrophoresis showing the refolding of OmpA, several single Trp mutants of OmpA, and the N-terminal transmembrane domain (residues 1–176) of the Trp-7 mutant into micelles of C_8E_4.** Upon refolding, the apparent molecular mass of OmpA and the mutants shift from 35 to 30 kDa and that of the N-terminal transmembrane domain Trp-7-(1–176) shifts from 19 to 21 kDa. 11.4 μM OmpA was refolded by dilution into 18 mM C_8E_4 micelles at pH 10.0 and 40 °C. The samples were boiled or incubated at 40 °C in SDS for 5 min, as indicated. 10 μg of protein were loaded on each lane.

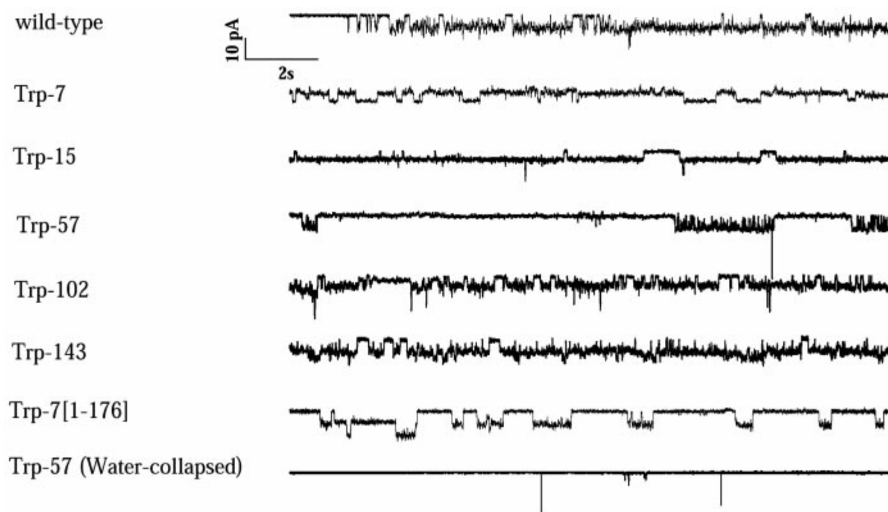

FIG. 3. **Single channel recordings of small channels of refolded OmpA, its single Trp mutants, and the N-terminal transmembrane domain (residues 1–176) of the Trp-7 mutant in planar bilayers.** Experimental conditions are the same as those described in the legend to Fig. 1. The trace of a control experiment with a misfolded, water-collapsed OmpA (Trp-57 mutant) is shown at the bottom of the figure.

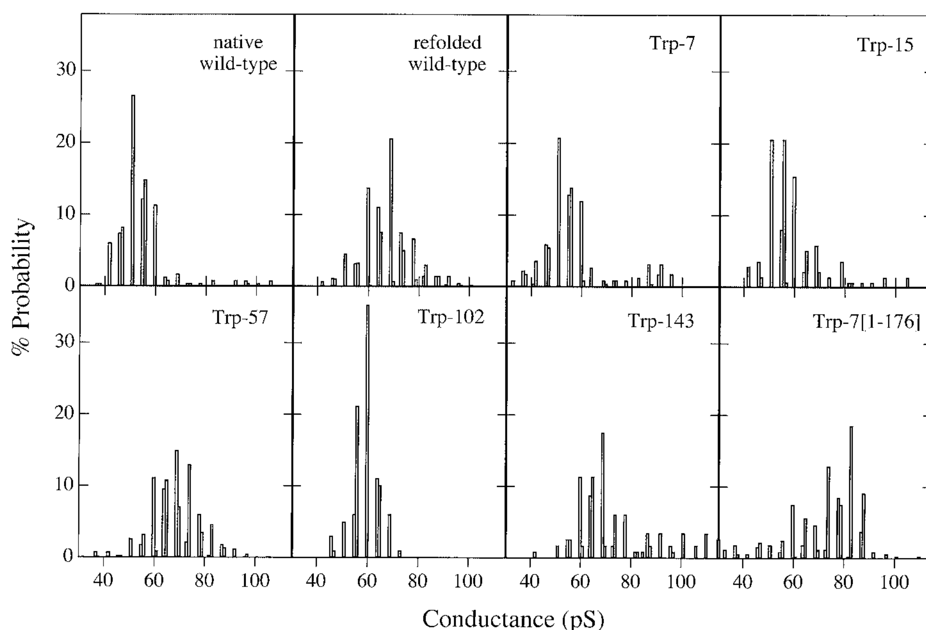

FIG. 4. **Histograms showing the distribution of small channels of refolded OmpA, its single Trp mutants, and the N-terminal transmembrane domain (residues 1–176) of the Trp-7 mutant in planar bilayers**. Channel activities were recorded at 100 mV in 1 M KCl buffer. The number of evaluated events and mean channel conductances for each protein are described under "Results."

conformations with relatively rare interconversions. For example, gramicidin A is known to form "mini-channels" and the interconversion frequency between the main and mini-channels is rather low (19). Analyzing 1173 small OmpA channel events from seven independent experiments, we found that their single channel conductance values ranged from 40 to 95 pS and followed an approximately Gaussian distribution with a mean value of 66 pS and a standard error of 15 pS. In all experiments, the membranes were stable, and the channel activity lasted for the duration of 15–30-min experiments. The large channels of refolded OmpA were relatively noisy in the open state (Fig. 5, *top trace*). Despite this noise, we were able to measure the distribution of the large channel events of the refolded OmpA as shown in the *lower left panel* of Fig. 5. The mean large channel conductance of refolded OmpA was 261 pS (212 events), *i.e.* similar to the conductance change associated with the small-to-large channel conversion of native OmpA (see Fig. 1).

Channel Activities of Single Tryptophan Mutants of OmpA—OmpA contains five tryptophans that are each located on a different transmembrane strand of the N-terminal β-barrel. In previous work we have used single Trp mutants of OmpA to monitor the site-specific rates of polypetide translocation and thus were able to dissect the folding pathway of this protein in lipid bilayers at unprecedent resolution (13). These mutants each had four of the five native tryptophans replaced by phenylalanines. To support our earlier conclusion, which was based on the absence of a perturbation of a phage-binding epitope, that these mutations did not significantly perturb the overall structure and function of OmpA, we wanted to know whether these changes had an effect on the single channel conductance of refolded OmpA. Fig. 2 shows that all five single Trp mutants that were used in our previous study (13) refolded into C_8E_4 micelles as determined by the gel shift assay. Representative recordings of small channels of each of these refolded mutant proteins are shown in *traces 2–6* of Fig. 3. Although there are clear differences in open channel noise levels and average open times, similar single channel conductance levels were observed for all mutants and these were not much different from those of the wild-type protein. The Trp-7 mutant exhibited well defined channels, which, however, were slightly smaller in conductance than the wild-type channels. Analyzing 217 events yielded a mean channel conductance of 51 pS (Fig. 4). The channels of the Trp-15 mutant were typically open for several seconds and closed only for very short durations. The 136 counted events exhibited a mean channel

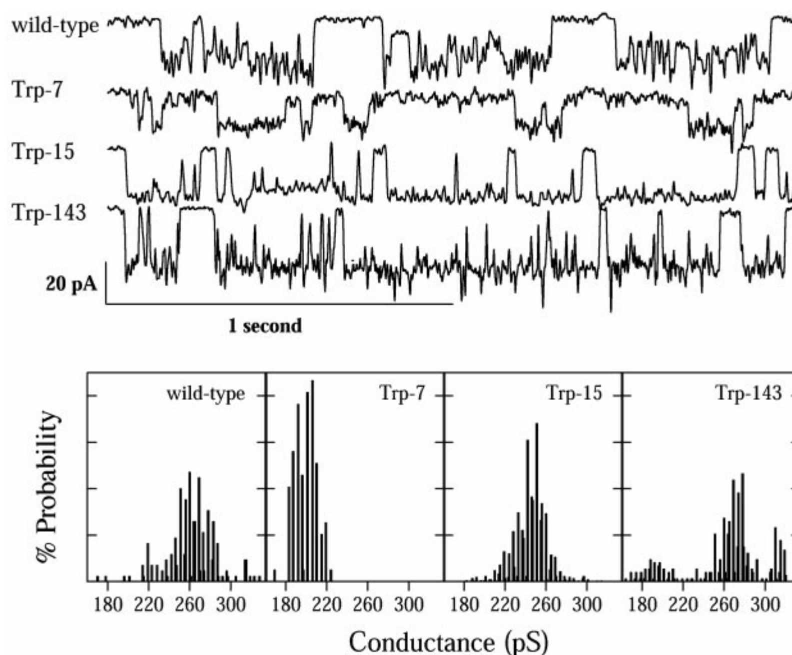

FIG. 5. **Single channel recordings and histograms of large channels of refolded OmpA and some of its single Trp mutants in planar lipid bilayers.** *Top,* four representative recordings showing large channels formed after incorporation of refolded OmpA and single Trp mutants. Experimental conditions are the same as described in the legend to Fig. 1. *Bottom,* histograms showing the distribution of the large channel openings and closings of refolded OmpA and the single Trp mutants Trp-7, Trp-15, and Trp-143. The number of evaluated events and mean channel conductances for each protein are described under "Results."

conductance of 54 pS (Fig. 4). A different pattern was observed with the Trp-57 mutant. The channels were either closed or open for several seconds, but while in their open state, they showed fast fluctuations which may correspond to rapid opening and closing events. For 647 events counted, the mean channel conductance was 67 pS. The channels formed by the Trp-102 and Trp-143 mutants displayed a similar behavior, but were open most of the time (Fig. 3). The 624 events counted for Trp-102 had a conductance of 59 pS, whereas the mean channel conductance of Trp-143 was 65 pS, counted for 114 events (Fig. 4). Thus, the mean channel conductance levels were about 60–70 pS for the refolded wild-type, Trp-57, Trp-102, and Trp-143 proteins and about 50–60 pS for native OmpA and the refolded Trp-7 and Trp-15 proteins.

Large channels were also observed for all refolded single Trp mutants. For example, Trp-15 exhibited a mean conductance of 246 pS (1040 events) and Trp-143 a mean conductance of 270 pS (365 total >150 pS events; Fig. 5). In addition, relatively rare conductance steps of intermediate size (mean 191 pS) were observed with this mutant. Trp-7, Trp-57, and Trp-102 also showed intermediate size single channel conductance levels (only Trp-7 is shown as an example in Fig. 5). Channels of the order of 198 pS conductance (98 events), 155 pS (42 events), and 161 pS (70 events) were observed for the Trp-7, Trp-57, and Trp-102 mutants, respectively. Fig. 3 (*bottom trace*) shows the recording of a further control that was carried out with the Trp-57 mutant protein. In this case, the protein was deliberately misfolded. It is well known that OmpA hydrophobically collapses, exhibits a very different CD spectrum, and eventually aggregates when diluted from an 8 M urea solution into an aqueous buffer lacking detergent micelles or lipid bilayers (16, 23). When an equivalent amount of water-collapsed OmpA was added to the planar bilayers, neither small nor large single channel events could be observed. This experiment demonstrates that the channels observed in the *upper traces* of Figs. 3 and 5 are because of functionally refolded OmpA.

The N-terminal Domain of OmpA Forms Only Small Channels—Because OmpA is a two domain protein consisting of an N-terminal transmembrane β-barrel domain and a periplasmic C-terminal domain, it is interesting to ask whether the transmembrane domain alone exhibits a similar channel activity as the full-length protein. To address this question we expressed the N-terminal domain (residues 1–176) of the Trp-7 mutant protein, designated Trp-7-(1–176). Trp-7-(1–176) was correctly targeted to the outer membrane of *E. coli,* which gives a first indication that this domain may fold and assemble normally in the outer membrane. The purified fragment was then refolded in C_8E_4 and analyzed by its migration on SDS gels, as were the full-length proteins. Interestingly, upon renaturation the fragment exhibited an upwards rather than a downwards shift on the gel: the heat-denatured unfolded protein ran at an apparent molecular mass of 19 kDa, whereas the refolded protein ran at a larger apparent molecular mass of 21 kDa (Fig. 2, *last two lanes*). Similar results have been reported previously for similar fragments of OmpA (21, 22). When the refolded N-terminal domain was incorporated into DPhPC/*n*-decane bilayers using the same protocol as for the full-length protein, single channel events were again observed (Fig. 3, *second trace from the bottom*). These single channel conductance steps were very well defined and exhibited less baseline noise than the corresponding full-length protein. Occasionally two channels were observed to be open simultaneously. For 318 counted events, the mean unitary channel conductance was 78 pS (Fig. 4), *i.e.* 27 pS larger than that of the corresponding full-length protein. Larger channels were never observed with this protein.

DISCUSSION

We have investigated the single channel activity of the outer membrane protein A of *E. coli*, several single tryptophan mutants of this protein, and its N-terminal β-barrel transmembrane domain in planar lipid bilayers. Whether purified as a native protein by detergent extraction from outer membranes or refolded from an unfolded form that was purified by extraction in 8 M urea, OmpA formed small (~60 pS) and large (~260 or ~320 pS) channels in DPhPC bilayers in 1 M KCl and at an applied potential of 100 mV. The 60 pS channels were more frequent and possibly precursors of the 260/320 pS channels. For some, but not all single Trp mutants, intermediate channels with conductance levels of ~170 pS were also observed. In contrast, the N-terminal transmembrane domain of OmpA exhibited only small channels with a unitary conductance of ~80 pS in 1 M KCl and at a 100-mV applied potential.

All conductance levels that have been measured with OmpA lie between those of the gramicidin A channel (21.1 pS (24)) and

the channel formed by the matrix porin OmpF (800 pS (5)) in planar lipid bilayers under comparable experimental conditions. The atomic structures of these two proteins are known, and their single channel ion conductivities have been studied extensively. The gramicidin A channel is formed by a tube-like structure, consisting of a N-to-N β-helix dimer, with a fairly uniform inner diameter of about 3.5 Å (25). The outer surface of this structure is hydrophobic and is properly positioned in the lipid bilayer by two rings of tryptophans that reside at the two C-terminal ends of the tube. These features of the gramicidin channel are therefore similar to those of the much larger porins and OmpA whose structures are also characterized by two belts of aromatic side chains at the ends of the barrels and within the polar headgroup region of the lipid bilayer. The channel-forming motif of the trimeric matrix porin OmpF of *E. coli* is a 16-stranded β-barrel with a large central pore (1). An extended β-hairpin folds into the lumen of the OmpF barrel and forms a constriction, which restricts the cross-section of the pore to 11 by 7 Å in the center of the bilayer. The exclusion limit of OmpF for the passage of uncharged molecules is about 600 Da. Based on these comparisons and our measured single channel conductance levels, one can estimate that the pore diameters of the OmpA channels should lie between about 3 and 10 Å. This estimate must be considered very crude because the correlation between pore size and single channel conductance behavior of β-barrel proteins is poor (5, 6).

The larger conductance channels of OmpA are similar to the 280 and 360 pS channels that have been observed with OprF of *Pseudomonas aeruginosa* and *P. syringae* in 1 M KCl (26, 27). These proteins are closely related to OmpA. For comparison, the maltose-specific porin, LamB, has a single channel conductance of only 160 pS in 1 M KCl (29, 30), and another outer membrane protein, Omp43 of *Wolinella recta,* exhibits a single channel conductance of 490 pS in 1 M KCl (28). Although LamB forms an 18-stranded β-barrel, its channel diameter is reduced to 5 Å by a large β-hairpin that partially fills the lumen of the pore (2).

Sugawara and Nikaido (9, 10) measured the permeability of OmpA that was reconstituted into liposomes with a series of uncharged solutes. They concluded that OmpA exists in two conformations in lipid bilayers, one that is permeable to molecules up to 600 Da and an other that is essentially impermeable to neutral solutes. In the preparation of native OmpA that we used about 15–20% of the protein was estimated to be in the larger pore conformation (15). This estimate is somewhat lower than the relative frequency of the larger ion channels that we observed with the native protein (~45%) but is in excellent agreement with the frequency of traces with the larger channels that were formed by the refolded OmpA and its single Trp mutants (see "Results"). Therefore, we tentatively correlate our 260/320 pS channel with the large pore conformation previously described by Sugawara and Nikaido (9, 10). In an earlier study, Saint *et al.* (8) reported a single channel conductance of 180 pS for OmpA. Their 180 pS channels measured in 0.25 M KCl may correspond to our 260/320 pS channels measured in 1 M KCl; experiments were conducted at a potential of 100 mV in both cases. Surprisingly, these authors did not observe the smaller channels of OmpA that we observed very consistently. In 0.25 M KCl, these channels would exhibit conductances near 15 pS and therefore may have escaped detection.

These results on the channel function seem collectively incompatible with the recently determined crystal structure of the transmembrane domain of OmpA. In this structure the interior of the β-barrel is quite densely packed with hydrophilic residues, which are cross-connected with an intricate hydrogen-bonding network and some salt bridges (7). Although several water-filled large cavities occur within the lumen of the barrel, they do not form a continuous passage for water or ions. Moreover, the salt bridge formed between residues Glu-52 and Arg-138 and flanked by Phe-40 and Tyr-94 constitutes a major constriction of the pore. As noted by Pautsch and Schulz (7), conductance of ions by this structure is difficult to explain. Because we observe single channel activity not only with full-length OmpA but with the expressed and refolded transmembrane domain, we suggest that at least some of the OmpA molecules assume more dynamic structures in lipid bilayers than in the protein-detergent co-crystals. Although unlikely, an alternative formal possibility is that the three peripheral single site mutations that were introduced into OmpA for crystallization purposes (7) altered the structure and channel behavior of the transmembrane domain of OmpA.

An interesting observation was made with OprF of *Pseudomonas fluorescens*. Depending on whether the protein was isolated from the outer membrane of cells that were grown at 8 or 28 °C, single channel conductances of 80 or 260 pS, respectively, were recorded in planar bilayers in 1 M NaCl (31). In addition, the lower growth temperature form of OprF was completely digested by trypsin within 1 h, but the higher growth temperature form was protected from trypsin digestion. Based on these results, the authors suggested that the two conductance states could represent two different conformations of OprF; the smaller channel may conduct ions through the 8-stranded β-barrel transmembrane domain, whereas the periplasmic domain may become more intimately associated with the membrane in the larger channel conformation. Because we observed frequent small, but no large channels with the expressed N-terminal domain of OmpA, it is possible that the C-terminal domain of OmpA participates in the formation of the larger channel in a similar fashion.

Apart from re-examining the single channel conductance of OmpA, a further goal of this study was to determine (*a*) whether refolded OmpA exhibited a similar activity as native OmpA and (*b*) whether multiple substitutions of tryptophans by phenylalanines affected this activity of OmpA. Refolded OmpA reproduced the smaller and larger channels of native OmpA quite accurately; the small channels conducted at 52 ± 15 and 66 ± 15 pS and the large channels at 270/320 and 261 ± 30 pS for the native and refolded proteins, respectively. The mean small channel conductances of the five single Trp mutants of OmpA were 51, 54, 67, 59, and 65 pS (Fig. 4), and those of the larger channels were 198, 246, 155, 161, and 270 (Fig. 5) for Trp-7, Trp-15, Trp-57, Trp-102, and Trp-143, respectively. Obviously, the small channel conductances (and therefore presumably also the corresponding conformations) are not significantly different for the native, the refolded wild-type, and all five single Trp mutant OmpA proteins. The same argument holds true for the large channel conformation of the native, the refolded wild-type, and the Trp-15 and Trp-143 mutant OmpA proteins. The Trp-7, Trp-57, and Trp-102 mutant OmpA proteins exhibited channels of intermediate instead of the larger conductance level, which may or may not represent a different C-terminal domain conformation. In any case, the fact that the wild-type and all mutant OmpAs formed small channels of the same conductance levels as the native protein provides strong support that the N-terminal domain has refolded into its native structure. Specifically the Trp substitutions, which are all confined to the N-terminal domain, appear to alter neither the refolding nor the single channel conductance level induced by this domain.

Our finding that the large channels form only in the presence of the C-terminal domain establishes a new functional assay for

the proper refolding of this domain of OmpA. In fact, these results present the first experimental evidence that the entire OmpA protein, including the C-terminal domain, is functionally refolded by our refolding protocol. All previous evidence for the refolding of OmpA was deduced from biochemical and spectroscopic similarities between the native and refolded forms of the protein, *i.e.* assays that largely measure the folding of the N-terminal β-barrel transmembrane domain. Despite this success, there are two as yet unexplained differences between the native and refolded proteins: the open channel noise is larger in the refolded than in the native proteins and the dynamic interconversion between the small and large channels seems to be largely blocked in the refolded proteins. These differences may be because of differences of the *E. coli* strains and purification protocols that were used or to a small amount of dodecylmaltoside that was present in the native OmpA preparations. Further experimentation will be required to resolve these issues.

In conclusion, OmpA forms channels of two or, perhaps, three conductance levels in planar lipid bilayers. The smaller channel can be clearly associated with the β-barrel transmembrane domain, but the larger channels also require the C-terminal domain. The measurement of large channels therefore provides a new assay for the folding of this latter domain. Extrapolating from the studies with the related OprF protein, the C-terminal domain may associate more tightly with the membrane in the large channel conformation. The two channel conformations are probably interconvertible, but the kinetics of interconversion are presently only poorly understood. It is possible that the larger channel conformation is induced by specific detergents, lipopolysaccharide, and an applied membrane potential. Although OmpA clearly functions as a pore in reconstituted planar bilayers or liposomes, it is not clear whether this is its real physiological activity in the outer membrane of *E. coli*. The N-terminal β-barrel domain may simply function as a membrane anchor for the periplasmic domain, which may have as yet undefined functions *in vivo* (32, 33). Notwithstanding these reservations, OmpA now serves also as a model for an ion channel with a well defined 8-stranded β-barrel structure. The protein further serves as a model for studying the folding of integral membrane proteins. The packing of residues within the channel may now be studied by protein engineering, which is important to understand the stability of a (inside-out) membrane protein, membrane protein folding, and the mechanism of ion conductance.

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