The Outer Membrane Protein OmpW Forms an Eight-stranded \( \beta \)-Barrel with a Hydrophobic Channel*

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Escherichia coli OmpW belongs to a family of small outer membrane proteins that are widespread in Gram-negative bacteria. Their functions are unknown, but recent data suggest that they may be involved in the protection of bacteria against various forms of environmental stress. To gain insight into the function of these proteins we have determined the crystal structure of E. coli OmpW to 2.7-Å resolution. The structure shows that OmpW forms an 8-stranded \( \beta \)-barrel with a long and narrow hydrophobic channel that contains a bound \( n \)-dodecyl-N,N-dimethylamino-N-oxide detergent molecule. Single channel conductance experiments show that OmpW functions as an ion channel in planar lipid bilayers. The channel activity can be blocked by the addition of \( n \)-dodecyl-N,N-dimethylamino-N-oxide. Taken together, the data suggest that members of the OmpW family could be involved in the transport of small hydrophobic molecules across the bacterial outer membrane.

The outer membrane (OM) of Gram-negative bacteria is a protective barrier that hinders the permeability of both hydrophilic and hydrophobic compounds, because of the presence of lipopolysaccharide (LPS) within the outer leaflet of the OM (1). To obtain nutrients and other molecules that are necessary for growth and function of the cell, Gram-negative bacteria have channels within their OM that facilitate uptake of these molecules. With respect to the transport of small, hydrophilic substances, these channels can be divided in three classes, based on their mode of transport (1): general porins, substrate-specific transporters, and active transporters. A wealth of structural and functional information is available for many of these OM channel proteins, which form monomeric or trimeric barrels that are each composed of 12–22 anti-parallel \( \beta \)-strands. In addition to OM proteins with established transport functions, the OM also contains a considerable number of smaller, monomeric \( \beta \)-barrels that are composed of 8 or 10 \( \beta \)-strands. These proteins have been implicated in a wide range of functions including OM lipid metabolism, cell adhesion, and structural functions. One of these small OM proteins is OmpA from Escherichia coli, which belongs to a protein family with a number of established and putative functions, the most important of which is to provide structural stability to the cell via interactions of its C-terminal domain with the periplasmic peptidoglycan layer (1). Another member of the small OM protein family is NspA from Neisseria meningitidis, which belongs to the Opa family of proteins that are thought to mediate adhesion to host cells (2).

A fundamental question is whether these small barrels can function as transport channels. Arguing against this possibility are the crystal structures that have been determined for several of these proteins, and which do not show continuous channels that would be consistent with transport functions. On the other hand, it has been shown that, at least in vitro, OmpA forms both small and large ion channels (3) and is permeable to larger uncharged solutes (4), suggesting that the static pictures provided by the crystal structures may be misleading.

The OmpW/AlkL family (5) is a family of small OM \( \beta \)-barrel proteins for which some, albeit indirect, evidence for transport exists. The proteins belonging to this family are widespread in Gram-negative bacteria, have a length of about 200–230 amino acids, and are predicted to form 8-stranded \( \beta \)-barrels. One member of this family, OmpW from Vibrio cholerae, was first described more than 20 years ago and has attracted interest for vaccine development, as it is very immunogenic and seems to be present in all known V. cholerae strains (6–8). E. coli OmpW, which under normal growth conditions is expressed in only minor amounts, is a receptor for colicin S4 (9), but otherwise nothing is known about its function. Interestingly, E. coli OmpW shows 27% sequence identity to AlkL from Pseudomonas oleovorans, which is located in an operon that is dedicated to the degradation of alkanes by this organism (10). A similar degree of sequence identity is observed between E. coli OmpW and NahQ/DoxH, which are proteins of the naphthalene catabolic pathway operon that is present in several Pseudomonas species (11). These sequence similarities suggest an as of yet unproven role for OmpW in the transport of various hydrophobic molecules across the OM. A putative, albeit ill-defined, transport function is also suggested by the fact that growth of Vibrio species in the presence of high concentrations of NaCl results in a dramatic (10–20-fold) induction of OmpW, implying an involvement of this protein in osmoregulation (12, 13).

The lack of a high-resolution structure of any of the OmpW/AlkL family proteins has so far hindered attempts to assign more specific functions to members of this OM protein family. To address the question of whether OmpW homologues could form channels for the transport of small (hydrophobic) molecules, we have determined the x-ray crystal structure of OmpW from E. coli and performed single-channel conductance experiments of OmpW in planar lipid bilayers. The results suggest that members of the OmpW/AlkL family form channels for the transport of small (hydrophobic) compounds.

**EXPERIMENTAL PROCEDURES**

Protein Expression and Purification—The gene for E. coli OmpW, including the signal sequence and with a hexa-histidine tag following the C-terminal phenylalanine residue, was amplified by PCR from genomic DNA, and cloned into the pB22 vector (14), which is under

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The atomic coordinates and structure factors (code 2FIT (trigonal) and 2FIT (orthorhombic OmpW)) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 Supported by National Institutes of Health Grant GM074624 and a PEW scholarship.

2 The abbreviations used are: OM, outer membrane; LDAO, \( n \)-dodecyl-N,N-dimethyl-amine-N-oxide; LPS, lipopolysaccharide; SIRAS, single isomorphous replacement with anomalous scattering; DPPC, dipalmitoylphosphatidylcholine.
control of the arabinose promoter (15). OmpW was expressed in C43 (DE3) cells (genotype F−ompTGal hsdSB(rB mB) dcm lon ADE3 pLys-S and two uncharacterized mutations described in Ref. 16) grown in 2× YT medium by induction with 0.2% arabinose for 3 h at 37 °C. After harvesting by centrifugation, the cells were resuspended in TSB buffer (20 mM Tris-HCl, 300 mM NaCl, 10% glycerol, pH 8) and ruptured by two passes at 15,000–20,000 psi in a microfluidizer (Avestin Emulsiflex C-3). Total (inner and outer) membranes were collected by centrifugation at 131,000 × g and solubilized by homogenization in 1% n-dodecyl-
N,N-dimethylamine-N-oxide (LDAO) in TSB buffer, followed by stirring of the suspension at 4 °C for 30 min. Following centrifugation at 131,000 × g, the membrane extract derived from 12 liters of cells (A600 = 1.0) was loaded onto a 10-ml nickel affinity column (Chelating Sepharose; Amersham Biosciences) equilibrated with 0.2% LDAO in TSB. The nickel column was washed with 20 volumes of buffer containing 5 mM imidazole and eluted with 200 mM imidazole. OmpW was further purified by gel filtration on a Superdex-200 26/60 column (Amersham Biosciences) in 10 mM sodium acetate, 50 mM NaCl, 10% glycerol, 0.05% LDAO, pH 5.5. This step was followed by cation exchange on a Resource-S column (Amersham Biosciences) equilibrated in 10 mM sodium acetate, 50 mM NaCl, 10% glycerol, 0.45% tetraethylene-mono-
octylether (C8E4), pH 5.5, and applying a linear NaCl gradient to 0.5 M. In addition to C9E9, OmpW was also purified by cation exchange in 0.05% LDAO and 1% β-octylglucoside. The pure protein (final yield 5–10 mg per 12 liters of cells) was concentrated to 5–10 mg/ml and flash frozen in liquid nitrogen.

Sucrose Gradient Centrifugation—C43 (DE3) cells expressing OmpW were ruptured as described above in 20 mM Tris-HCl, 0.5 M EDTA, pH 7.5. Cell debris was removed by centrifugation at 10,000 × g for 30 min, followed by collection of total membranes as described above. Membranes were resuspended in 4 ml of buffer and layered on a step gradient of 35–60% sucrose in buffer, with 5% increments of sucrose (10 ml each). Following centrifugation at 131,000 × g for 18 h, the two membrane fractions were drawn off, solubilized in 1% LDAO, and purified by nickel affinity chromatography as described above. OmpW was located in the heavier outer membrane fraction located at the 50–55% sucrose interface (data not shown).

Crystallization and Data Collection—Crystallization trials of OmpW purified in LDAO, β-octylglucoside, and C9E9 were set up at 295 K by mixing 0.8 μl of protein solution with 0.8 μl of reservoir solutions from commercially available crystallization screens (Crystal Screen I from Hampton Research and The Classics and MbClass II screens from Nxtal Biotechnologies). Whereas crystals proved difficult to obtain in β-octylglucoside and LDAO, OmpW purified in C9E9 crystallized readily under various conditions. After optimization, two crystal forms were obtained that diffracted to sufficient resolution to allow collection of x-ray data. The first of these (referred to as “trigonal”) crystallized as diamonds in space group P321 from 28 to 32% PEG400, 0.2 M CaCl2, and 50 mM glycine, pH 9, and contained 3 molecules in the asymmetric unit (Vm ~2.8 Å³/Da, corresponding to ~56% solvent content). The diffraction from these crystals was highly anisotropic, with diffraction limits ranging from 2.4 to 3.6 Å. The second crystal form (referred to as “orthorhombic”) crystallized as bipyramids in space group P212121 from 27 to 33% MPD, 0.2 M ammonium acetate, 50 mM citrate, pH 5.6, and contained 6 molecules in the asymmetric unit (Vm ~3.2 Å³/Da, corresponding to ~62% solvent content). The diffraction of these crystals was more isotropic and extended to 2.7 Å resolution. The diffraction patterns of the orthorhombic crystals could also be indexed and integrated in higher symmetry lattices (primitive cubic (P23), rhombohedral (R3), and primitive tetragonal (P41)), but subsequent scaling resulted in an unacceptable (5–20%) number of rejections and high χ² values. Both crystal forms were frozen directly from the mother liquor in liquid nitrogen for data collection. Native and derivative data were collected at NSLS beamlines X25 and X6A at Brookhaven National Lab. All diffraction data were processed with HKL2000 (17).

Heavy Atom Derivatization, Phasing, and Refinement—Despite extensive efforts, selenomethionine-substituted protein did not yield crystals that diffracted to high resolution. Consequently, we had to solve the OmpW structure by multiple isomorphous replacement methods. Because co-crystallization approaches were not successful, a large number of heavy atom compounds were soaked into the trigonal crystals (which were obtained prior to the orthorhombic crystals). Several of the heavy atom compounds (K2PtCl4, OsCl3) resulted in derivatives that were severely non-isomorphous with respect to the native crystals, and single anomalous dispersion and single isomorphous replacement with anomalous scattering (SIRAS) approaches did not produce useful phases. A useful derivative was eventually obtained by soaking the crystals in 2–5 mM YbCl3 for ~3 h. A single anomaly dispersion dataset at the ytterbium peak wavelength (1.38 Å) was recorded for a derivatized crystal that diffracted to 3.2 Å resolution. Heavy atom sites (three in total; one for each molecule) were identified using SOLVE (18). Phases were obtained by SIRAS and refinement of the heavy atom sites within SHARP (19). The resulting electron density maps (after phase extension to 3.0 Å) differed greatly in quality for the three OmpW molecules within the asymmetric unit, presumably because of the anisotropy in the crystals. Initially this allowed only two of the three OmpW molecules in the asymmetric unit to be built manually, using O (20). During the course of refinement in CNS (21), the third molecule could be built as well, although its B-factors were much higher than those of the other two molecules. The orthorhombic crystal form was solved by molecular replacement with the program PHASER (22) implemented in ccp4 (23), using the trigonal OmpW structure as a search model. A clear solution with 6 molecules in the asymmetric unit was obtained. The model was refined in CNS as described above. During refinement of both crystal forms, it was necessary to impose tight NCS restraints on the protein to obtain acceptable Rfree values. The data collection and refinement statistics are summarized in Table 1. Both OmpW crystal forms have good stereochemistry. Only one residue lies within the disallowed regions of the Ramachandran plot. This residue (Ser175) is present in the least well defined molecule in the asymmetric unit of trigonal OmpW (Table 1), and is located in surface loop L4 in a region with weak electron density.

Single-channel Conductance Experiments—Samples for single-channel recordings in planar lipid bilayers were prepared in two ways: 1) by 100-fold dilution of concentrated (8–10 mg/ml) detergent-solubilized protein in 20 mM C9E9 buffer (OmpW: pH 5.5, 10 mM potassium acetate; OmpA: pH 9.3, 10 mM glycine; Tsc: pH 8.0, 10 mM Tris-HCl) containing 0.5 M EDTA, and 2) by detergent removal and reconstitution into dipalmitoyl phosphatidylcholine (DPPC) proteoliposomes. For this, 80–100 μg/ml of detergent-solubilized (OmpW, Tsc) or refolded (OmpA) protein prepared by method 1 was mixed with DPPC to give a final lipid concentration of 10 mM. More C9E9 was added until the solutions became clear. The mixtures were dialyzed for 72 h (with 4 solution changes) against the corresponding detergent-free buffer. 5 μl of protein in C9E9 micelles or DPPC proteoliposomes were added to the cis- and trans-compartments of a bilayer setup for single-channel recordings. The two compartments, each containing 1.5 ml of 1 M KCl buffer (1 mM KCl, 10 mM Tris-HCl, pH 7.3) and a chloride silver electrode, were separated by a preformed DiPhPC/decane planar bilayer in a 500-μm hole in a Teflon partition. The cis-compartment was grounded while the potential was applied to the trans-compartment. For titration
with ligands, channels were prepared by adding 5 μl of OmpW solution (200 μg/ml) reconstituted into 10 mM DPPC to both compartments. Small aliquots of 1 mM arabinose or 0.25 mM LDAO stock solutions were alternately added to the cis- and trans-compartments, followed by mixing and application of +140 or −140 mV potentials. No attempts were made to reconstitute the protein unidirectionally or to determine the effect of unilateral additions of the ligands. The current fluctuation across the bilayer was filtered at 100 Hz with an 8-pole low-pass filter and recorded digitally at 1 kHz using the LABMAN program. Single channel conductance events were analyzed interactively using IGOR software (Wave-metrics, Portland, OR) (3).

### RESULTS

#### Description of the Structure

The final tridimensional OmpW model contains three protomers, three glycerol, eight C$_8$E$_4$, and three LDAO molecules in the asymmetric unit. The entire OmpW molecule is remarkably hydrophobic, and no polar or charged residue comes within 4 Å of the bound detergent. Instead, only the hydrophobic character of the interior of the barrel, as shown in Fig. 2, is apparent. The apolar character of the barrel interior extends about 30–35 Å inward-pointing residues of the barrel wall, only 20 are hydrophilic. The apolar character of the interior of the barrel, as shown in Fig. 2, is apparent. The most remarkable feature in the OmpW structure is the hydrophobic character of the interior of the barrel, as shown in Fig. 2. Of the 62 inward-pointing residues of the barrel wall, only 20 are hydrophilic (Ser, His, Asp, Gla, Asn, Gln, Arg, and Lys) and the rest are hydrophobic. The apolar character of the barrel interior extends about 30–35 Å from the extracellular exit to almost the middle of the membrane (Fig. 2), where Leu$_{56}$ in strand S3 and Trp$_{155}$ in strand S7 form a “hydrophobic gate” (Fig. 3). The interior of the barrel on the periplasmic side of the gate has a more hydrophilic character, comparable with that found in most other OM proteins (Fig. 2). In this hydrophilic vestibule region, salt bridges are likely present between the residues pairs Glu$_{54}$–Arg$_{5}$, Arg$_{5}$–Glu$_{5}$, and Arg$_{190}$–Glu$_{5}$.

In the tridimensional crystals, an elongated stretch of density is present in the center of the hydrophobic channel at the extracellular side (Fig. 3A). In the tridimensional crystals this density, although present, is much weaker and not continuous, and for this reason we have not built anything at this site in the tridimensional crystal form. The length of the density is consistent with an LDAO detergent molecule, which was used during the purification of the protein (see “Experimental Procedures”). The environment of the putative LDAO molecule is remarkably hydrophobic, and no polar or charged residue comes within 4 Å of the bound detergent. Instead, only the hydrophobic character of the interior of the barrel, as shown in Fig. 2, is apparent. The apolar character of the barrel interior extends about 30–35 Å from the extracellular exit to almost the middle of the membrane (Fig. 2), where Leu$_{56}$ in strand S3 and Trp$_{155}$ in strand S7 form a “hydrophobic gate” (Fig. 3). The interior of the barrel on the periplasmic side of the gate has a more hydrophilic character, comparable with that found in most other OM proteins (Fig. 2). In this hydrophilic vestibule region, salt bridges are likely present between the residues pairs Glu$_{54}$–Arg$_{5}$, Arg$_{5}$–Glu$_{5}$, and Arg$_{190}$–Glu$_{5}$.

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TABLE 1

| Summary of data collection and refinement statistics | Trigonal | Native | Orthorhombic |
|--------------------------------------------------|---------|--------|--------------|
| Wavelength (Å)                                   | 1.38    | 1.10   | 1.10         |
| Resolution range (Å, 3.3–3.2)                    | 5.0–3.2 | 5.0–3.0| 4.0–2.7      |
| Space group                                      | P3$_2$1 | P3$_2$1| P2$_2$2$_1$  |
| Unit cell parameters (Å, °)                       | 82.0, 82.0, 186.3 | 82.2, 82.2, 186.2 | 118.7, 118.8, 118.5 |
| Mosaicity (%)                                     | 0.39    | 0.45   | 0.39         |
| $R_{merge}$ (%) (outer shell)                     | 16.3 (47.8) | 7.6 (37.5) | 8.7 (52.9)  |
| Completeness (%) (outer shell)                    | 94.5 (63.4) | 98.8 (99.0) | 99.5 (100.0) |
| $F_{calc}/F_{obs}$ (outer shell)                  | 17.8 (2.4) | 34.0 (6.1) | 25.7 (4.2)  |
| IRAS phasing power after SHARP (isometric/ano)    | 1.08/1.37| 1.08/1.37| 1.08/1.37   |
| IRAS FOM after SHARP (centric/acentric)           | 0.32/0.41| 0.32/0.41| 0.32/0.41   |

$R_{merge} = \Sigma|F_{calc} - |F_{obs}||/\Sigma|F_{calc}|$ for the 95% of reflection data used in refinement.

$R_{free} = \Sigma|F_{calc} - |F_{obs}||/\Sigma|F_{calc}|$ for the remaining 5%. 

$\%$ Residues in regions of Ramachandran plot

| Trigonal | Native | Orthorhombic |
|---------|--------|--------------|
| YbCl$_3$ | 8.0–3.0 | 8.0–2.7 |
| Total no. atoms (non-hydrogens)                 | 8521    | 8586        |
| R$_{merge}$$^a$/$R_{free}$$^a$ (%)              | 27.4/32.8 | 29.3/31.4 |
| r.m.s. deviation$^b$ bond lengths (Å)/bond angles (°) | 0.0085/1.60 | 0.0095/1.63 |
| Average B-factor (Å$^2$)                        | 70.1 (57.0) | 42.0        |
| LDAO                                             | 105.6 (80.3)$^c$ | 56.2        |
| Glycerol                                         | 61.6 (56.8)$^c$ | 56.2        |
| C$_8$E$_4$                                       | 88.6 (92.3)$^c$ | 56.2        |
| % Residues in regions of Ramachandran plot       | 96.0/0.2 (97.3/0.0)$^d$ | 100.0/0.0 |

$^a$ The values between parentheses are for the best defined molecule within the asymmetric unit.

$^b$ The values between parentheses are for the best defined molecule within the asymmetric unit.

$^c$ The values between parentheses are for the best defined molecule within the asymmetric unit.

$^d$ The values between parentheses are for the best defined molecule within the asymmetric unit.

$^e$ The values between parentheses are for the best defined molecule within the asymmetric unit.
phobic residues Phe31, Ile65, Val76, Leu123, Leu126, Leu128, Tyr165, and Val176 are close (within 4 Å) to the hydrophobic tail of the LDAO molecule (Fig. 3B). The polar head group of the LDAO molecule is located at the end of the barrel, and is solvent accessible (Fig. 3A). Because the LDAO molecule is still bound to OmpW after detergent exchange to C8E4 (see “Experimental Procedures”), it is likely to bind in the channel with relatively high affinity.

In the periplasmic vestibule, a significant (~5 σ) patch of density is present at identical positions in the 2Fo − Fc maps of both the trigonal and orthorhombic crystals (Fig. 3C). Both hydrophobic (Phe7, Tyr88, Leu143, and Met46) and charged residues (Glu5, Glu2, Arg9, Arg190, and Asp141) contribute to this binding pocket (Fig. 3C). Although it is not possible to identify this density unambiguously at the current resolution, we have assigned it as a molecule of glycerol for several reasons. The binding pocket is similar in character to glycerol binding sites in other proteins (for example, GlpF; Ref. 25). The hydroxyl groups of the putative glycerol molecule are close (between 2.2 and 2.8 Å) to the carboxyl groups of Glu5, Glu2, and Asp141, whereas the glycerol carbon backbone is stacked against the aromatic ring of Phe7 (Fig. 3C). Supporting this assignment is the fact that inclusion of glycerol during refinement resulted in a slightly lower (0.2%) Rfree value. This contrasts with the inclusion of a water molecule or several different cations at this site, which resulted in somewhat increased values of Rfree. It should be noted that an arabinose molecule (and probably other pentose sugars as well) is also compatible with the density at this site, giving similar Rfree values as for glycerol. However, because both OmpW crystal forms were purified and crystallized in the presence of 10% glycerol, the density in the binding pocket is more likely to correspond to glycerol than to arabinose.

**Single-channel Conductance Measurements**—Single-channel conductance recordings of OmpW reconstituted into planar lipid bilayers are shown in Fig. 4. Similar traces are observed when the protein is reconstituted from C8E4 micelles or DPPC proteoliposomes. A statistical analysis shows that OmpW reconstituted from C8E4 has a conductance of 17 ± 8 pS (mean ± S.E.) at 140 mV in 1 M KCl (determined from 279 events in two independent experiments). When reconstituted from DPPC, the conductance of OmpW is 19 ± 8 pS (determined from 629 events in four experiments) (Fig. 5). Under identical conditions, OmpA has a conductance of 60–70 pS independent of the method of reconstitution (Figs. 4 and 5). These values for OmpA are similar to those reported before (3), and correspond to the formation of a small channel by the 8-stranded β-barrel.
of the N-terminal ∼176 residues of OmpA. For comparison, we also measured the single-channel conductance of the nucleoside transporter Tsx reconstituted from C8E4 micelles. This protein has recently been shown to form a 12-stranded β-barrel with a narrow central pore (26), which displays one of the smallest single-channel conductance values of any OM protein (27). The single-channel conductance of Tsx, measured under the same conditions as OmpW and OmpA is 10 ± 4 pS (Figs. 4 and 5), similar to the value previously reported for this protein under the same electrolyte conditions (27).

Guided by the extra features of density present in the crystal structure, we titrated several potential ligands to DPPC-reconstituted OmpW in planar lipid bilayers to observe their effect on the conductivity of this channel. When glycerol was added up to a concentration of 135 mM (∼1.2%), no clear change in single-channel conductance behavior was observed (data not shown). The use of higher glycerol concentrations, such as those used during purification and crystallization, was not feasible because such concentrations compromised the stability of the planar lipid bilayers. By contrast, the titration of arabinose up to 20 mM resulted in an inhibition of the channel activity, as shown in Fig. 6. Analyzing several minutes worth of recording data, the open probability decreased from 33 to 9% after addition of 20 mM arabinose. It is likely that arabinose binds at the site where glycerol may be bound in the OmpW structure. At this stage, however, it is not clear if the binding of glycerol, arabinose, or related compounds to OmpW has functional significance, because the polar vestibule residues that are likely involved in the binding of such ligands are not well conserved between OmpW homologues (Fig. 7).

When LDAO was added to OmpW, its channel activity was efficiently blocked at micromolar concentrations (Fig. 6). The open probability decreased to 0.4% after addition of 5 μM LDAO. This result supports our assignment of the electron density present in the extracellular part of the channel in the trigonal crystals, and reinforces our notion that LDAO binds to the channel with high affinity.

DISCUSSION

OmpW Contains a Deep Hydrophobic Binding Pocket—The most interesting feature of the OmpW channel is the hydrophobic character of the major part of the barrel interior, as shown in Fig. 2. This is very unusual for OM proteins (which typically have barrel interiors that are hydrophilic and filled with water), and suggests functional significance. So far, the only known small OM proteins that have hydrophobic pockets within their barrels are the Neisserial cell adhesion protein NspA and the lipid A palmitoyltransferase FagP, the structures of which were recently solved by x-ray crystallography and NMR (2, 28–30). Both proteins have hydrophobic pockets that are occupied by a detergent molecule. The hydrophobic pocket in NspA is relatively shallow,
located on the extracellular side at the top of the barrel, and thought to be involved in adhesion to host cell lipids. The enzyme PagP has a hydrophobic pocket inside the barrel similar to that of OmpW, although the pocket in OmpW is substantially longer. The hydrophobic pocket in PagP is thought to bind a fatty acid molecule for the subsequent transfer to lipid A during lipopolysaccharide synthesis in the OM. Consistent with this notion is the gap in the PagP barrel wall, which is thought to provide room for the lateral entry and exit of the fatty acid in and out of the active site in exchange with the outer leaflet of the OM (28–30). By analogy with these two proteins, we consider it likely that the hydrophobic pocket of OmpW where the LDAO molecule is bound serves as a binding site for molecules that have a hydrophobic character. This notion is reinforced by the fact that the hydrophobicity of the eight residues that are located within 4 Å of the LDAO molecule is conserved.

FIGURE 4. Representative single channel recordings of OmpW, OmpA, and Tsx in planar diphytanoyl-PC lipid bilayers in 1 M KCl at pH 7 and +140 mV. Proteins were reconstituted from C8E4 detergent micelles or DPPC (DpPO) proteoliposomes as indicated.

FIGURE 5. Histograms for the single-channel conductance distributions of OmpW, OmpA, and Tsx in planar lipid bilayers. The reconstitution method, total number of the identified opening events, and the mean ± S.E. conductance values are annotated in each histogram. In the histogram at the lower right, the conductance distributions of OmpW, OmpA, and Tsx reconstituted from C8E4 are compared directly.
between OmpW homologues, including members of the AlkL family (Fig. 7).

Whereas it seems clear that OmpW proteins can bind hydrophobic molecules that are present in the extracellular environment, it is still an open question whether OmpW, and indeed any member of the small OM protein families, could function as transporters \textit{in vivo}. The previously determined crystal structures of the N-terminal domain of OmpA, as well as the current structures of OmpW, do not show a continuous channel spanning the entire membrane. In OmpA, this is because of the presence of a number of hydrogen bonds and salt bridges formed by polar and charged residues that line the lumen of the channel. In the case of OmpW, a continuous apolar channel that is likely to be large enough for the passage of small molecules extends all the way from the extracellular surface to the putative hydrophobic gate formed by Leu$^{56}$ and Trp$^{155}$, located approximately halfway through the membrane (Figs. 3 and 8). On the periplasmic side of the gate, interactions between polar and charged residues are responsible for the closed state of the OmpW channel observed in the crystal structures. However, previous results (3) and the present single channel data for OmpW and OmpA strongly suggest that these proteins form ion channels \textit{in vitro}. As argued before (3), this indicates that the interiors of these proteins are much more dynamic than is evident from the static x-ray crystallographic structures, which do not show continuous channels. In accord with this notion, conformational exchange on the $\mu$s to ms time scale of several barrel residues of OmpA has been observed by NMR (31). Molecular dynamics simulations also suggest that the side chains of residues in the putative channel of OmpA are mobile enough to, at least transiently, allow the formation of a transport pore (32). Thus, whereas OmpA has several functions that are not transport-related, accumulating data suggest that it may be involved in transport as well.

**OmpW Forms a Hydrophobic Channel**—The single channel conductance of OmpW in 1 M KCl and at $\pm 140 \text{ mV}$ is about 18 pS. This is a three to four times smaller value than that of OmpA under the same conditions (Fig. 5). The smallest OM protein that is known to form a \textit{bona fide} transport channel is the nucleoside transporter Tsx, which has a 12-stranded $\beta$-barrel with a small central pore that crosses the entire width of the membrane (26). Interestingly, under conditions identical to those employed for OmpW and OmpA, we measured a single channel...
conductance value of only 10 pS for Tsx (Fig. 5), confirming results from a previous study (33) and demonstrating that the permanently open pore of Tsx gives rise to smaller conductance values than the transient pores of OmpA and OmpW. The very small conductance values measured for the nucleoside transporter provide, in our opinion, a strong argument that the larger conductance measured for OmpW and OmpA are the result of the formation of bona fide channels by these proteins.

The much lower ion permeability of the OmpW relative to the OmpA channel is likely because of the lining of the interior of the OmpW channel with hydrophobic residues as opposed to hydrophilic residues, which would make the passage of ions energetically more favorable in the case of OmpA. In addition, there may also be differences in pore diameter or side chain dynamics between the two channels.

Recent data for OmpW homologs suggest that members of this protein family may be involved in bacterial adaptation in response to various stress conditions. Proteomic analysis of Stenotrophomonas sp. OK-5 revealed that OmpW is, together with stress-shock proteins such as DnaK and OsmC, one of the few proteins that become significantly induced by the presence of trinitrotoluene in the growth media (34). In another report, the expression of OmpW from Vibrio cholerae was found to be dependent on in vitro culture conditions such as temperature, salinity, and availability of nutrients or oxygen (35).

Although these data imply a transport function for OmpW family proteins, further studies are needed to fully understand their role in bacterial adaptation.
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members, it is clear that such a broad regulation of protein expression by a number of external stress factors hampers efforts to assign more specific functions to these proteins. As an example, based on the observation that OmpW expression was dramatically decreased in a ceftriaxone-resistant strain of Salmonella typhimurium, it was recently proposed that OmpW might be involved in the uptake of this antibiotic (36). Based on our structure, however, it is unlikely that this relatively large (660 Da) and polar molecule would be able to traverse the OM through the OmpW channel.

Although the currently available data suggest that members of the OmpW family function as transporters under various stress conditions, the identities of the putative substrates that are transported by OmpW proteins are not yet clear. The hydrophobic character of the interior of the OmpW channel, the presence of LDAO in the crystal structure, and the blockage of the channel activity observed with LDAO, make small hydrophobic molecules the most likely transport substrates of OmpW channels. Moreover, although we cannot exclude a role for OmpW in the export of (toxic) molecules from the cell, the presence of a well-defined hydrophobic binding pocket on the extracellular side of the membrane makes an import function much more likely. The sequence similarity of OmpW and AlkL/NahQ further supports the role of OmpW proteins as transporters of hydrophobic molecules. However, it is clear that, even though AlkL may be involved in alkane transport in Pseudomonas species, alkanes are only metabolized by certain species of Gram-negative bacteria. For this reason, OmpW channels are unlikely to function as general facilitators of alkane transport. So far, experiments designed to demonstrate the involvement of the putative alkane transporter AlkL from P. oleovorans in alkane uptake are inconclusive because alkI knock-out strains were able to grow on octane as the sole carbon source. However, this negative result can be explained by a redundancy in OM transport systems for these compounds. For example, members of the FadL family of long-chain fatty acid/xenobiotics transporters (37, 38) are present in all Pseudomonas species sequenced to date, and it is likely, although not yet proven, that these proteins can also transport hydrophobic molecules other than long-chain fatty acids, such as alkanes. In addition, alkI, like the other alk genes, is plasmid encoded, and a chromosomally encoded OmpW homologue is likely to exist in P. oleovorans. Therefore, future experiments designed to demonstrate involvement of OmpW/AlkL family proteins in the uptake of alkanes or other hydrophobic molecules should be carried out in a fadI/ompw knock-out strain.

A Possible Mechanism for Transport of Hydrophobic Compounds by OmpW—As has been shown for long-chain fatty acids (39), amphiphilic and hydrophobic molecules readily pass through regular phospholipid bilayers, making dedicated transport systems in principle not necessary for such membranes. However, the bacterial OM is an unusual biological membrane in the sense that it contains LPS as the principal component in the outer leaflet (1, 37). The sugar moieties of the LPS form a polar layer on the outside of the cell, which is the reason why amphiphilic and hydrophobic compounds do not easily pass through that layer. By contrast, the hydrocarbon region of the OM is unlikely to present a major permeability barrier for these compounds. Taking this into consideration, the current crystal structure suggests an intriguing transport model. OmpW possesses a continuous passageway that leads from the extracellular surface where the LDAO molecule is bound, to a gap that is present in the barrel wall between residues Leu56–Phe61 of strand S3 and residues His77–Pro81 of strand S4 (Figs. 1B and 8). Importantly, the gap is located close to the extracellular belt of aromatic residues (not shown), likely placing it at the hydrophilic/hydrophobic interface of the outer leaflet of the OM. The gap as present in the structure seems wide enough to allow for a lateral passage of small hydrophobic molecules into the lipid bilayer. It is notable that three proline residues, Pro60, Pro80, and Pro81, are located in the immediate vicinity of the barrel gap. Although only Pro80 is absolutely conserved between OmpW homologues, the other two prolines are also fairly well conserved and are present in the majority of OmpW sequences (Fig. 7). These prolines may help to stabilize the unusual conformation of strands S3 and S4 in this region, preventing the formation of interstrand hydrogen bonds and enabling the formation of a gap in the barrel wall. Thus, hydrophobic molecules could bypass the polar part of the LPS layer by diffusing down the hydrophobic channel and moving laterally through the gap in the barrel wall into the OM. Substrate molecules could then diffuse or flip to the inner layer of the OM, followed by diffusion into and across the periplasm, possibly assisted by periplasmic binding proteins. The alternative to this lateral exit model would be a more classical mechanism, in which the substrate could diffuse down the entire length of the barrel to

* J. van Beilen, unpublished results.
reach the periplasm. In this transport mechanism, the substrate would need to pass through the vestibule on the periplasmic end of the OmpW channel (Fig. 2), which is filled with polar side chains, some of which form salt bridges in our structure. However, the observed single channel activities of OmpW, together with the fact that the channel activity is blocked by a substrate that likely binds in the vestibule, shows that polar molecules such as ions traverse the OmpW barrel in the classical way. Site-directed mutagenesis experiments, for example, targeting the proline residues in the vicinity of the barrel gap or eliminating the salt bridges in the polar vestibule, could provide a means to distinguish between the lateral exit model and the classical transport model. To do this, however, transport substrates for the OmpW channels will have to be identified first, and this will be the focus of future experiments.

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