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Membrane Topology of the Outer Membrane Protein OprH from *Pseudomonas aeruginosa*: PCR-Mediated Site-Directed Insertion and Deletion Mutagenesis

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The 21-kDa outer membrane protein OprH from *Pseudomonas aeruginosa* is overexpressed under Mg^{2+}-starvation conditions and when overproduced causes resistance to polymyxin B, gentamicin, and EDTA. By circular dichroism analysis, OprH revealed a calculated β-sheet structure content of 47.3%. PCR-based site-directed deletion and epitope insertion mutagenesis was used to test a topological model of OprH as an eight-stranded β-barrel. Three permissive and seven nonpermissive malarial epitope insertion mutants and four permissive and four nonpermissive deletion mutants confirmed the general accuracy of this model. Thus, OprH is the smallest outer membrane protein to date to be confirmed as a β-stranded protein.

Overproduction of the *Pseudomonas aeruginosa* outer membrane protein OprH, either as a result of growth of this organism in Mg^{2+}-deficient (20 μM) medium or because of genetic mutation, leads to resistance of cells to EDTA and polycationic antibiotics, such as polymyxins and aminoglycosides (2). A variety of data favor the hypothesis that this basic 178-amino-acid protein replaces the divalent cations that normally cross bridge adjacent lipopolysaccharide (LPS) molecules and consequently blocks both the self-promoted uptake of these polycations and the action of EDTA on the outer membrane (2, 16, 31). Self-promoted uptake normally involves the competitive displacement of divalent cations from their surface LPS binding sites by the polycationic antibiotics, because of their far higher affinity for such sites. This results in a destabilization of the outer membrane and the subsequent penetration of these antibiotics through the destabilized outer membrane. To the best of our knowledge, the ability of OprH to block trans-outer membrane uptake of antibiotics is unique, and the comparison of its sequence with those in databases has revealed no proteins with substantial homology. In addition, no outer membrane protein with less than 300 amino acids has been structurally characterized to date. Therefore, we were interested in defining the structural features and membrane topology of OprH.

Two basic types of procedures have been used to date to characterize the structure of bacterial outer membrane proteins. The optimal method is fine-structure definition by X-ray crystallography. Although outer membrane protein crystals are rather difficult to solve because of phase problems, one specific porin (21) and four nonspecific porins (7, 29) have been structurally defined to form, respectively, 18- and 16-stranded β-barrels with short periplasmic turns and large loops that are arrayed on the surface or fold into the channel. A second procedure involves prediction of the membrane topology of outer membrane proteins rich in β-structure by a combination of evaluation of turn-promoting residues and definition of transmembrane amphipathic stretches (1, 4, 11, 12, 15, 17). This procedure can yield membrane topology models with surprising accuracy compared with crystal structures of the same protein (7, 21). Such models can be tested by the apparently correct assumption that the regions of these proteins that loop out from the β-strands can accept both deletions and insertions, whereas the transmembrane β-strands cannot (1, 11, 12, 25). To date, a variety of porin molecules, including specific (4, 11) and nonspecific (1) porins, gated porins (5, 6, 13), and porins of the OmpA superfamily (18, 30), have been modeled and tested by insertion mutagenesis or, less often, by deletion mutagenesis. All such porins show thematic similarity but rarely show strong sequence alignment (12). Therefore, we were interested to see how a substantially smaller outer membrane protein, OprH, without known porin activity (32) related to those porins characterized to date. The data presented here indicate that despite its smaller size, unique sequence, and nonporin function, OprH appears to be structurally related to the porins.

OprH was purified from outer membranes (8) of the OprH-overproducing polymyxin B-resistant mutant *P. aeruginosa* H181 (2) by a modification of the methods of Bell and Hancock (3), involving solubilization with 10 mM Tris-Cl–50 mM EDTA–3% (wt/vol) *n*-octyl-polyoxyethylene (Bachem Bioscience Inc.) (pH 8.0) and anion exchange chromatography with a MonoQ column and fast protein liquid chromatography (3).

OprH was subjected to analysis by circular dichroism, as described previously (23). The spectra obtained could be used to predict a secondary structure comprising 47.3% β-sheets, 15.7% α-helices, 13.6% random coils and 24.4% β-turns. Because of this high β-structured content and the lack of hydrophobic stretches of sufficient length to traverse the membrane, a topology model was predicted on the basis of those principles used for the porins (12).

According to this model (Fig. 1), OprH was presented as a β-barrel consisting of eight antiparallel amphipathic β-strands, spanning the outer membrane and connected by three β-turns (T1 to T3) at the periplasmic side and four longer loops (L1 to L4) exposed the cell surface. The loops generally corresponded to the hydrophilic maxima in the protein, when analyzed according to the method of Hopp and Woods (10), whereas the periplasmic turns contained residues with strong β-turn pro-

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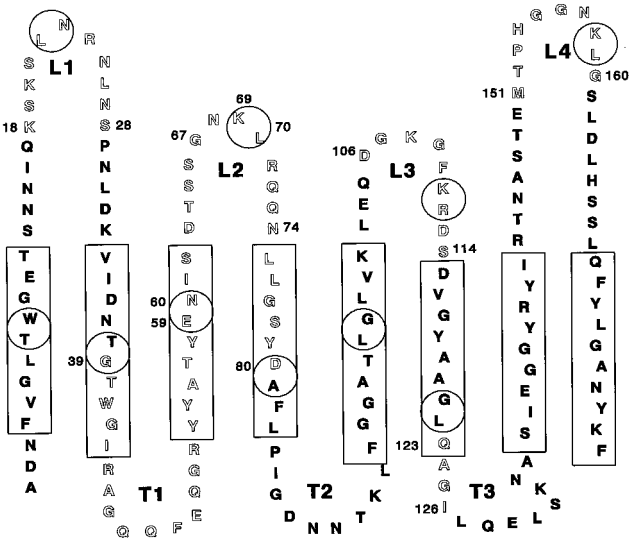

FIG. 1. Topological model of OprH in the outer membrane. The boxes enclose the proposed transmembrane β-strands, and the model is oriented so that the cell-surface-exposed loops are facing up. Circles indicate sites at which the malarial epitope was inserted. The amino acid residues deleted in various mutants are presented as unfilled letters, with the consecutive set of deletions between amino acids 39 and 80 being delineated by the amino acid numbers (Table 1). External loops are labeled L1 to L4, and periplasmic β-turns are labeled T1 to T3.

pensity, as analyzed according to the method of Paul and Rosenbush (17). The predicted amphipathic β-strands generally contained alternating polar and nonpolar residues (9 to 10 residues) of lengths that were in agreement with the lengths of β-strands obtained for the crystallographically defined porin structures (6, 26). The topological model of OprH predicted 45% β-sheets, in good agreement with the obtained circular dichroism spectra (47.3% β-sheets). Recently, a new alignment strategy for porins based on amphipathicity was published (15). Three potential β-strands were predicted to be in the OprH sequence, corresponding to no. 2, 7, and 8 in our model. The most C-terminal β-strand of OprH showed substantial homology to the most C-terminal (16th) β-strand of the porin superfamily (12), a result consistent with our interpretation of OprH as being a β-barrel.

The model was tested by PCR-based site-specific deletion and insertion mutagenesis. General molecular genetic procedures were performed according to the method of Sambrook et al. (20). To construct an expression system allowing controlled overexpression of the *oprH* gene, the coding region of the previously cloned (3) *oprH* gene was amplified by PCR and inserted into the *Nde*I and *Bam*HI sites of vector pT7-7, using the provided ribosome binding site and the T7 promoter (27). The resulting plasmid, pBHR720, enabled strong overexpression of *oprH* in *Escherichia coli* BL21 (DE3), and recombinant OprH was localized in the outer membrane.

Site-directed epitope insertion mutagenesis was done by applying two separate PCRs. One reaction served to amplify the N-terminal DNA fragment of *oprH* and to introduce, at the proposed insertion site, an *Eco*RI restriction site. The other reaction served to amplify the C-terminal part of the *oprH* coding region, again introducing an *Eco*RI restriction site at the insertion site as well as a DNA region encoding the amino acid sequence KRKNPNANPN. The malarial epitope (NPNA NPN) was derived from the circumsporozoite protein of the human malarial parasite *Plasmodium falciparum* (30). The KRK motif preceding this epitope was used to ensure that insertions into β-strands would interrupt their amphipathic nature and thus disrupt the correct folding of the protein. Prior to the start codon and after the stop codon of the *oprH* coding region, the restriction sites *Nde*I and *Bam*HI, respectively, were introduced. Both PCR products were gel purified, properly restricted, and together inserted into the *Nde*I and *Bam*HI sites of the vector pT7-7 (26). To perform site-specific deletion mutagenesis, the overlap extension method of Ho et al. (9) was employed. One deletion was done by removing a 12-bp *Pst*I fragment from the *oprH* coding region. All mutants were confirmed by DNA sequencing.

Oligonucleotide primers for these mutagenesis procedures were generally designed by using the PCGENE computer program to minimize nonspecific binding and primer-dimer interactions. Their actual sequences can be obtained from the authors of the present study. These primers were synthesized on an Applied Biosystems Inc. model 392 DNA/RNA synthesizer.

When these procedures were used, 10 epitope insertion mutants and 8 deletion mutants were constructed in the *oprH* gene (for the locations of these mutations, see Fig. 1 and Table 1). To test for expression in both *E. coli* and *P. aeruginosa*, the *Xba*I-*Bam*HI fragments from pBHR720 and its derivatives were inserted into the corresponding sites of the broad-host-range vector pUCP19 (22). The resulting plasmids, pBHR20 and derivatives, allowed expression (from the *lac* promoter, using the ribosome binding site derived from the vector pT7-7), of *oprH* and its variants in *E. coli* CE1248 and *P. aeruginosa* H703 (Fig. 2; Table 1).

Epitope insertions in predicted loops L1 (expressed from plasmid pBHR23), L2 (pBHR29), and L4 (pBHR38) were per-

TABLE 1. Expression and characterization of the OprH epitope insertion and deletion mutant proteins

Plasmid	Amino acid insertion or deletion site[a]	Expression by SDS-PAGE[b]	Apparent molecular mass (kDa)	
			Untreated	Trypsin digested
pBHR20	Wild type	+++	19.5	17
pBHR21	::7	−		
pBHR22	Δ18–28	++	19	19
pBHR23	::22	+++	21	17
pBHR24	::38	−		
pBHR25	Δ39–59	−		
pBHR26	::59	−		
pBHR27	Δ60–69	−		
pBHR28	Δ67–74	++	19	17
pBHR29	::69	+++	19.5	17
pBHR30	Δ70–80	−		
pBHR31	::80	−		
pBHR32	::98	−		
pBHR33	Δ106–114	+	19	17
pBHR34	::111	−		
pBHR35	::121	−		
pBHR36	Δ123–126	−		
pBHR37	Δ151–160	+++	19	17
pBHR38	::158	+++	19.5	17

[a] Position 1 is the N-terminal amino acid of the mature OprH amino acid sequence. Insertion sites are indicated by double colons followed by the amino acid after which the insertion was done. Deletions are indicated by Δ and the actual residues removed.

[b] +++, level comparable to that of wild-type OprH; ++ and +, reduced levels compared with that of wild-type OprH; −, undetectable level. Outer membrane protein preparations from *E. coli* CE1248 carrying the given plasmids were used. Similar data were obtained for the OprH variants expressed in *P. aeruginosa* cells. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

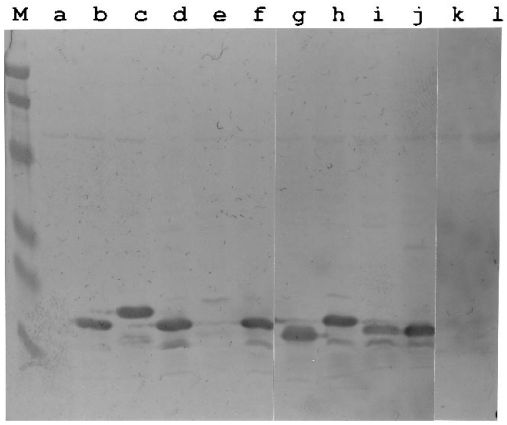

FIG. 2. Western immunoblot (28) of outer membrane proteins from *E. coli* CE1248, containing various plasmids, after induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). *E. coli* CE1248 cells contained plasmid pUCP19 (negative control) (lane a), pBHR20 (expressing wild-type OprH) (lane b), pBHR23 (OprH::22) (lane c), pBHR29 (OprH::69) (lane d), pBHR34 (OprH::111) (lane e), pBHR38 (OprH::158) (lane f), pBHR22 (OprHΔ18-28) (lane g), pBHR28 (OprHΔ67-74) (lane h), pBHR33 (OprHΔ106-114) (lane i), pBHR37 (OprHΔ151-160) (lane j), pBHR26 (OprH::59) (lane k), or pBHR30 (OprHΔ70-80) (lane l). The blot was reacted with anti-OprH serum. Small differences in the apparent molecular weights of the mutants may relate to differences in the degree of unfolding (since OprH is a heat-modifiable outer membrane protein). Lane M, prestained molecular weight markers (from the top): 110,000, 84,000, 47,000, 33,000, 24,000, and 16,000.

missive, as demonstrated by localization of the corresponding gene products in the outer membranes (8) of *E. coli* CE1248 (Fig. 2) and *P. aeruginosa* H703 cells (data not shown). All of the other insertion sites were nonpermissive and were located in the predicted β-strands, except the insertion in L3 (pBHR34). Correspondingly, deletions were only structurally tolerated in the predicted loop regions L1 to L4 (expressed from plasmids pBHR22, pBHR28, pBHR33, and pBHR37, respectively), and these OprH mutant proteins cofractionated with the outer membranes in both *E. coli* (Fig. 2) and *P. aeruginosa* cells. The deletion constructed by removal of an internal *Pst*I fragment and affecting predicted β-turn T3 (pBHR36) was nonpermissive when studied under the same conditions as the above-described mutants, but when overexpressed with the T7 expression system, a faint band could be visualized in immunoblots of whole-cell lysates. Also, T7-based overexpression of deletion mutants affecting predicted β-strands 2 and 3 from plasmids pBHR25 and pBHR27 led to the formation of inclusion bodies, as demonstrated by the strong enrichment of these OprH mutant proteins during differential centrifugation (data not shown). All of the permissive mutants cloned into pUCP19 (pBHR20 derivatives) showed similar expression levels in *E. coli* and *P. aeruginosa* cells, except pBHR33, which encoded an OprH variant with an 8-amino-acid deletion in loop L3 close to the predicted β-strand region, which was weakly expressed (Table 1). Interestingly, a loop L3 deletion of a much larger outer membrane protein, OprD, previously exhibited a similar weak expression (11).

All of the permissive OprH mutants were tested for their surface exposure and proper folding in the outer membrane. The epitope insertion mutants carried an additional trypsin-sensitive region in the inserted sequence, provided by the KRK motif preceding the actual malarial epitope region. Whole cells of *E. coli* CE1248 containing various plasmids expressing the permissive OprH mutants, as well as outer membrane preparations of the same cells, were subjected to trypsin hydrolysis (11, 30) with 0.1 mg of trypsin (TPCK [tolylsulfonyl phenylalanyl chloromethyl ketone] treated; Sigma Chemical Co., St. Louis, Mo.). No difference in the susceptibility of OprH to trypsin digestion in whole cells and in outer membrane protein preparations was observed. Thus, presumably any trypsin-sensitive sites were exposed on the cell surface. Among the mutants, the mutant with a 10-amino-acid deletion in loop L1 (pBHR22) was completely resistant to trypsin digestion, and the mutant with a 9-amino acid deletion in loop L3 (pBHR33) was completely digested to a 17-kDa fragment, whereas the other mutants and wild-type OprH were partially cleaved by trypsin to a fragment with an apparent molecular mass of 17 kDa (Table 1). Overall, these data were consistent with the hypothesis that the permissive mutants folded correctly in the outer membrane and were surface exposed.

The surface exposure of the epitope insertion mutant proteins was assessed by indirect immunofluorescence (14). All three permissive epitope insertion mutant clones revealed comparable expression levels, as indicated by the same levels of fluorescence observed microscopically, when a polyclonal anti-OprH serum (2), as the primary antibody, and a secondary fluorescent-tagged antibody were used (Table 2). To determine the surface exposure of the malarial epitope inserted into these OprH mutants, intact cells expressing the mutant proteins were reacted with a malarial-epitope-specific monoclonal antibody (30) followed by a fluorescein isothiocyanate-conjugated secondary antibody and visualized under a fluorescence microscope. All three insertion mutants showed significant fluorescence compared with the negative control and the wild-type OprH-expressing clone (Table 2). Exceptionally high levels of fluorescence were observed with the mutant carrying the epitope insertion in loop L1, indicating that loop L1 was more readily surface accessible (Table 2).

The above-described data are consistent with the presented OprH model (Fig. 1) and indicate that OprH may form a β-barrel structure in the outer membrane, as is known to be the case for the porins. In contrast to the porins, however, OprH showed no channel-forming activity in lipid bilayer experiments (2). It is, nevertheless, possible that OprH, in addition to its role in blocking self-promoted uptake, forms a gated pore which is normally closed unless activated by a certain molecule, as proposed for FhuA, the Fe^{3+}-ferrichrome receptor protein from *E. coli* (5, 19). OprH has only eight β-strands, half the number observed for the nonspecific porins. Although the N terminus of *E. coli* outer membrane protein OmpA is supposed to have eight β-strands (18), a more recent model has suggested a total of 16 β-strands (24), consistent with the related *P. aeruginosa* porin OprF (23). Furthermore, there is no strong homology between OmpA/OprF and OprH. OprH

TABLE 2. Indirect immunofluorescence labeling of intact *E. coli* CE1248 cells containing various plasmids

Plasmid	Fluorescence[a] with:	
	Anti-OprH serum[b]	Anti-malarial-epitope monoclonal antibody
pUCP19	−	−
pBHR20 (wild-type OprH)	++	−
pBHR23 (OprH::22)	++	+++
pBHR29 (OprH::69)	++	+
pBHR38 (OprH::158)	++	+

[a] The values represent the fluorescence level ranging from no fluorescence (−) to strong fluorescence (+++).

[b] The anti-OprH serum was partially purified by twice adsorbing it with intact *E. coli* CE1248 cells.

is, therefore, by far the smallest of the β-stranded outer membrane proteins described to date, and we are intrigued by its strong structural similarity to other outer membrane proteins. Currently, we are functionally characterizing the OprH mutants to see if they can shed light on structure-activity relationships in this protein.

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