Structural Basis for the Interaction of Lipopolysaccharide with Outer Membrane Protein H (OprH) from Pseudomonas aeruginosa* S

Received for publication, July 8, 2011, and in revised form, August 17, 2011 Published, JBC Papers in Press, August 24, 2011, DOI 10.1074/jbc.M111.280933

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Background: Pseudomonas aeruginosa outer membrane protein OprH has been hypothesized to confer antibiotic resistance by interaction with LPS.

Results: The structure of OprH was solved and LPS interaction was demonstrated by solution NMR supported by pulldown and biochemical assays.

Conclusion: OprH forms a β-barrel in membrane and interacts with LPS in vivo and in vitro.

Significance: Structure and lipid interactions may help understand antibiotic resistance.

Pseudomonas aeruginosa is a major nosocomial pathogen that infects cystic fibrosis and immunocompromised patients. The impermeability of the P. aeruginosa outer membrane contributes substantially to the notorious antibiotic resistance of this human pathogen. This impermeability is partially imparted by the outer membrane protein H (OprH). Here we have solved the structure of OprH in a lipid environment by solution NMR. The structure reveals an eight-stranded β-barrel protein with four extracellular loops of unequal size. Fast time-scale dynamics measurements show that the extracellular loops are disordered and unstructured. It was previously suggested that the function of OprH is to provide increased stability to the outer membranes of P. aeruginosa by directly interacting with lipopolysaccharide (LPS) molecules. Using in vivo and in vitro biochemical assays, we show that OprH indeed interacts with LPS in P. aeruginosa outer membranes. Based upon NMR chemical shift perturbations observed upon the addition of LPS to OprH in lipid micelles, we conclude that the interaction is predominantly electrostatic and localized to charged regions near both rims of the barrel, but also through two conspicuous tyrosines in the middle of the bilayer. These results provide the first molecular structure of OprH and offer evidence for multiple interactions between OprH and LPS that likely contribute to the antibiotic resistance of P. aeruginosa.

Pseudomonas aeruginosa is the most common cause of pneumonia in cystic fibrosis patients (1). Pseudomonal infection in the cystic fibrosis lung results in antibiotic-resistant biofilms and is the leading cause of mortality in cystic fibrosis patients (2). It is also responsible for a majority of urinary tract and burn and wound infections and is the major nosocomial pathogen in hospital settings. The unusually high antibiotic resistance of Gram-negative P. aeruginosa is partially imparted by its extremely tight and stable outer membrane (OM), which makes infections with these bacteria clinically very difficult to treat (3). Additionally, the OM is densely packed with lipopolysaccharide (LPS), or endotoxin, that contributes substantially to biofilm formation in the pathogenesis of P. aeruginosa infections (4, 5). LPS molecules are found in the outer leaflet of the OM where they form a protective extracellular barrier against the penetration of potentially noxious molecules by divalent cation-mediated LPS-OM interactions (3). Displacement of divalent cations from LPS by polycationic antibiotics such as polymyxins and aminoglycosides through the self-promoted uptake pathway can lead to destabilization of the OM and increased susceptibility of these bacteria to antibiotics (6, 7). In laboratory settings, the increased susceptibility of P. aeruginosa to antibiotics caused by divalent cation displacement can be achieved by treatment with chelators such as EDTA.

OprH is genetically linked to the PhoP-PhoQ two-component regulatory system that is up-regulated in response to Mg2+-limited growth conditions (8, 9). As a member of the complete P. aeruginosa Mg2+-stimulon, the oprH-phoP-phoQ operon reinforces resistance to common antimicrobial cationic peptides such as polymyxin B and aminoglycosides (10, 11). Under Mg2+-deficient growth conditions, OprH is up-regulated and overexpressed so that it becomes a major component of the P. aeruginosa OM (8). Based on these correlations, OprH has been hypothesized to play a critical function in antibiotic resistance by acting as a surrogate for depleted Mg2+ ions on the outer membrane con

‡ The abbreviations used are: OM, outer membrane; OprH, outer membrane protein H; DHPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine; FhuA, ferric hydroxamate uptake A; TROSY, transverse relaxation optimized spectroscopy; HSQC, heteronuclear single quantum coherence; r.m.s., root mean square; r.m.s.d., root mean square deviation.

* This work was supported, in whole or in part, by National Institutes of Health Grant RO1 GM51329 (to L. K.T.). The atomic coordinates and structure factors (code 2LHF) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/). The NMR assignments reported in this paper were submitted to the Biological Magnetic Resonance Database under BMRB ID 17842.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–7.

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the surface of the cell, thus stabilizing the LPS network on the surface of the cell (12). This hypothesis is supported by evidence that OprH stabilizes the OM by increasing its protection from membrane perturbation and thereby increasing its antibiotic resistance (11). Although numerous studies have hinted at OprH-LPS interactions, direct evidence of these interactions has not been demonstrated previously.

OprH is a 21-kDa, 200-residue, basic (theoretical pI ~9.0) protein that is integral to the OM of P. aeruginosa. Based on hydrophathy analysis, OprH has been proposed to form a β-barrel with eight transmembrane strands and four extracellular loops (13). Circular dichroism data have further confirmed the β-sheet secondary structure of the protein. However, no high-resolution structure of OprH has yet been determined, and the molecular determinants of its postulated interaction with LPS remain elusive. Therefore, to better understand the contribution of OprH to the antibiotic resistance of P. aeruginosa, we have determined and evaluated its structure in a lipid environment in the presence and absence of LPS by solution NMR methods. We further show that LPS interacts directly with OprH in vitro and in vivo, and we identify interacting residues that could potentially be targeted in future studies aimed at further advancing our understanding of pseudomonal antibiotic resistance.

**EXPERIMENTAL PROCEDURES**

**FLAG Pulldown Assays**—An OprH deletion mutant strain of *P. aeruginosa* PA01 (PAO1ΔoprH) was created and transformed with the pHERD30T shuttle vector containing the OprH gene with a C-terminal FLAG tag, as described previously (14–16). Plasmid-containing strains with and without the cloned OprH gene were grown at 37 °C in M9 minimal medium with either 20 mM (low) or 500 mM (high) MgSO₄ for 8 h before induction with 2% arabinose. Cells were pelleted by centrifugation (20,000 × g, 15 min) 4–8 h after induction and resuspended in 50 mM Tris-HCl at pH 8.0, 300 mM NaCl, and 25% sucrose. Cells were lysed by sonication, and the insoluble pellets were washed in 50 mM NaPO₄ at pH 8.0, 300 mM NaCl, 20 mM imidazole, and 8 M urea and loaded onto a Ni²⁺-nitrilotriacetic acid Superflow (Qiagen) column by FPLC. OprH was eluted with elution buffer (50 mM NaPO₄ at pH 8.0, 300 mM NaCl, 20 mM imidazole, and 8 M urea) and loaded onto a Ni²⁺-nitrilotriacetic acid Superflow (Qiagen) column by FPLC. OprH was eluted with elution buffer (50 mM NaPO₄ at pH 8.0, 300 mM NaCl, 250 mM imidazol and 8 M urea). The purity was increased by running the elutant through a second Ni²⁺-nitrilotriacetic acid column.

**Refolding and Sample Preparation**—The refolding of OprH into 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) micelles followed the protocol previously described for *E. coli* OmpX with slight modification (17). Briefly, a sample containing 0.4 mM OprH in elution buffer was diluted 10-fold into 20 mM Tris-HCl at pH 8.5, 5 mM EDTA, and 0.6 mM l-arginine (refolding buffer) with 3% DHPC (Avanti Polar Lipids Inc). The refolding solution was incubated for 72 h at 37 °C before being dialyzed (20 min, room temperature) against 2.5 liters of 20 mM Tris-HCl at pH 8.5, 5 mM EDTA, and 50 mM KCl. The solution was concentrated, and the buffer was exchanged against 25 mM NaPO₄ at pH 6.0, 50 mM KCl, 0.05% NaN₃ with 5% D₂O by dilution/concentration. Final NMR samples were concentrated to 1.0–1.3 mM OprH and contained 150–175 mM DHPC as determined by ¹H NMR spectroscopy. Samples of OprH in DHPC:LPS mixed micelles were prepared by the addition of purified *P. aeruginosa* PA01 LPS (Sigma) from stock solutions directly to OprH-DHPC micelle samples. The LPS concentration in the stock solutions was determined by thioarbituric acid and Purpald assays for the measurement of 2-keto-3-deoxyoctonate (18).

**Trypsin Susceptibility Assay**—Trypsin from bovine pancreas (Sigma) was added from stock solutions to samples of unlabeled OprH in DHPC or DHPC:LPS micelles in 20 mM Tris-HCl at pH 7.3 with either 5 mM EDTA or 2 mM MgCl₂. After incubation at 37 °C for 5 h, SDS-PAGE sample loading buffer was added to trypsin-treated and untreated samples that were subsequently boiled or not boiled at 100 °C for 15 min. Samples were then used for SDS-PAGE on 15% Tris-glycine gels supplemented with 25 mM NaCl to increase band separation. Gels were stained with Coomassie Brilliant Blue R-250 (Pierce), and gel densitometry analysis was performed using the National Instruments LabVIEW 2010 software.

**NMR Spectroscopy**—All NMR experiments were recorded at 45 °C on a Bruker Avance III 800 spectrometer equipped with a triple-resonance cryoprobe. The one-dimensional TRACT...
pulsed scheme utilized to determine rotational correlation times has been previously described (19). All double- and triple-resonance experiments were from the Bruker Topspin version 2.1.6 software suite. For detergent/lipid screening and LPS studies, two-dimensional $^{15}\text{N}-^{1}\text{H}$ TROSY experiments were utilized. Sequential backbone assignments were obtained by recording TROSY versions of HNCA, HN(CO)CA, HN(CA)CB, HN(COCA)CB, HNCO, and HN(CA)CB experiments. Both $^{15}\text{N}-^{1}\text{H}$ TROSY and $^{15}\text{N}-^{1}\text{H}$ HSQC-NOESY-HSQC experiments with mixing times of 200 ms were recorded to obtain distance constraints. $R_1$, $R_2$, and [1H]–$^{15}\text{N}$ heteronuclear nuclear Overhauser effects (NOEs) were measured using two-dimensional $^{15}\text{N}-^{1}\text{H}$ TROSY-based experiments. NMR data were processed and analyzed with NMRPipe and Sparky software, respectively (20).

**Structure Calculation**—Distance constraints were calibrated and calculated based upon an average distance of 3.3 Å between β-strands using Cyana version 2.1 (21). Backbone dihedral angle constraints were determined from chemical shifts corrected for deuterium and TROSY effects using TALOS (22, 23). Hydrogen bond constraints derived from $^{1}\text{H}/2\text{H}$ exchange were obtained by recording TROSY versions of HNCA, HN(CA)CB, HN(COCA)CB, HNCO, and HN(CA)CB experiments. Both $^{15}\text{N}-^{1}\text{H}$ TROSY-based experiments. NMR data were processed and analyzed with NMRPipe and Sparky software, respectively (20).

**RESULTS**

**OprH Interacts with LPS in *P. aeruginosa* Outer Membranes**—Using FLAG pull-down assays from membrane lysates of *P. aeruginosa* bacterial strains containing a vector that expressed OprH with a C-terminal FLAG tag, we tested the hypothesis that OprH binds specifically with LPS in vivo. To do this, we first generated a mutant *P. aeruginosa* PAO1 strain that had the OprH gene deleted (PAO1ΔoprH) and transformed the mutant strain with either the empty pHERD30T vector as a control or the vector containing OprH-FLAG (pHERD30T-oprH-FLAG). Each strain was grown in minimal medium supplemented with limited concentrations of MgSO$_4$ (low Mg$^{2+}$), and PAO1ΔoprH (pHERD30T-oprH-FLAG) strains were additionally grown in minimal medium supplemented with high concentrations of MgSO$_4$ (high Mg$^{2+}$). After induction, the cell density of the cultures was normalized to maintain equivalent concentrations of membrane protein and LPS in all the final membrane lysates (Fig. 1, A and B). The lysates were then used for the immunoprecipitation of OprH-FLAG (Fig. 1, C and D). When OprH was precipitated from the membrane lysates of PAO1ΔoprH (pHERD30T-oprH-FLAG), a considerable amount of LPS was co-immunoprecipitated. The amount of LPS that was co-immunoprecipitated by OprH-FLAG from these cultures was also dependent on the concentration of Mg$^{2+}$ in the growth medium. Significantly less LPS was co-immunoprecipitated from the OM lysates of PAO1ΔoprH (pHERD30T-oprH-FLAG) cultures that were grown in high Mg$^{2+}$ when compared with low Mg$^{2+}$. When the OM lysates of PAO1ΔoprH (pHERD30T) were used for the immunoprecipitation, only trace amounts of LPS were co-immunoprecipitated, indicating that co-immunoprecipitation of LPS by OprH-FLAG was specifically due to low Mg$^{2+}$-enhanced binding of LPS to OprH in vivo.

**Protease Protection of OprH by LPS**—To directly demonstrate that LPS binds to OprH in vitro, the protein was expressed in *E. coli* and subsequently purified from inclusion bodies. This was achieved by deleting the membrane-targeting N-terminal signal sequence from OprH and attaching a C-terminal His$_6$ tag to facilitate protein purification. The resulting OprH–His$_6$-containing inclusion bodies were solubilized in 8 M urea and purified in an unfolded form. The purified protein was refolded using numerous detergents and lipids including dodecylphosphocholine, 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine, N,N-dimethyldodecylamine-N-oxide, and DHPC. Each detergent or lipid was tested for the ability to produce folded protein for biochemical analysis and NMR experiments.

The efficiency of refolding OprH was monitored by SDS-PAGE. Like many other OM proteins, the apparent molecular mass of OprH on SDS gels shifts when going from an unfolded to a folded form; the native protein runs at 18 kDa but shifts to 21 kDa after boiling in SDS-PAGE loading buffer (26, 27). We
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also noted that the addition of the His<sub>6</sub> tag causes a slight increase in the apparent molecular weight from these previously reported values. This reversible "heat modifiability" of the SDS-gel mobility of OM proteins has been used frequently as a reliable assay for their refolding. We found that OprH refolded most efficiently in the presence of the short chain lipid DHPC (supplemental Fig. 1). The quality and stability were further assessed by exchanging the refolded protein samples in different detergents or lipids into NMR buffer and acquiring $^{15}$N-$^1$H TROSY spectra at 45 °C (supplemental Fig. 2). Again, the best results in terms of efficiency, quality, and stability were found when OprH was refolded into DHPC micelles.

To assay for LPS binding to OprH in DHPC micelles in vitro, we exploited the arginine and lysine cleavage sites in the predicted extracellular loops of OprH. Each predicted extracellular loop has at least one potential trypsin cleavage site. After digestion of refolded OprH in DHPC micelles by trypsin, the apparent molecular weight of the protein on SDS gels before boiling the sample remained similar to the undigested protein (Fig. 2A). However, in contrast to the undigested protein, unfolding the trypsin-digested sample by boiling it in SDS loading buffer yielded several smaller protein fragments, the largest of which were ~8 and 5 kDa (Fig. 2A, lane 4). Therefore, the extracellular loops of OprH in DHPC micelles were accessible to trypsin digestion. When LPS was included in the micelles (1:125, LPS: DHPC molar ratio), some extracellular loops became protected from trypsin digestion as evidenced by the appearance of two protein bands with apparent molecular masses of ~18 and 15 kDa after boiling the mixed micelle protein sample (Fig. 2A, lane 8). A schematic diagram in Fig. 2A shows maps of the obtained cleavage products.

The addition of MgCl<sub>2</sub> to the digestion buffer resulted in complete reversal of this protease protection (Fig. 2A, lane 12). The relative fraction of each protected band was determined by gel densitometry and was found to be dependent on the LPS concentration in the mixed micelles (Fig. 2, B and C). The fraction of protected protein reached saturation at ~10-fold excess of LPS over OprH, at which point only 20% of the protein remained unprotected. To ensure that the trypsin protection of OprH was not a general consequence of adding charged lipids with increased acyl chain lengths (12-carbon versus 6-carbon for LPS and DHPC, respectively) to the protein-micelle complex, OprH in DHPC micelles was incubated with similar concentrations of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol. In each case, no protection from trypsin cleavage was observed (supplemental Fig. 3). Thus, protection of the extracellular loops of OprH results from specific binding to LPS and is dependent on the concentration of Mg<sup>2+</sup>.

Mass spectrometry of the 18-kDa LPS-protected band revealed that it consisted of an OprH fragment comprising residues Asn-26–His-187 (His<sub>6</sub> tag included) (data not shown). No signal was observed for residues Thr-13–Arg-25, and only a weak signal from a peptide corresponding to Ala-2–Glu-12 was observed. Therefore, the predicted extracellular loops 2, 3, and 4 of OprH were all at least partially protected from trypsin cleavage by the presence of LPS. In contrast, residue Arg-25 in predicted extracellular loop 1 was completely accessible to trypsin cleavage even in the presence of LPS. Based upon the molecular masses of the possible products resulting from tryp-
sin digestion of the extracellular loops, the 8- and 5-kDa fragments were identified as corresponding to Val-36–Lys-109 and Asp-114–Lys-159, respectively, which means that residues Lys-70 and Arg-72 in or near predicted loop 2 were protected from trypsin cleavage even in the absence of LPS.

Solution Structure of OprH in DHPC Micelles—To determine the structure of OprH by solution NMR, isotope-labeled OprH was produced by expressing the protein in E. coli that was cultured in minimal medium supplemented with 15N-(NH4)2SO4- and 2H-,13C-labeled glucose. The protein was then purified and refolded into DHPC as described above. The 15N-1H TROSY spectrum of 2H-,13C-,15N-labeled OprH in DHPC micelles displayed excellent dispersion of the backbone amide resonances from 7.5 to 9.5 ppm, with a slightly greater amount of peak overlap from 8.0 to 8.5 ppm (Fig. 3). 179 major cross-peaks could be identified. The overall correlation time of the OprH-DHPC micelle complex at 45 °C was determined to be 22 ns using one-dimensional TRACT (19) (supplemental Fig. 4). Based upon the correlation times of the OmpX-DHPC complex at 30 °C (60 kDa, 21 ns) (19) and the PagP-dodecylphosphocholine complex at 45 °C (50–60 kDa, 20 ns) (28), the molecular mass of the OprH-DHPC complex was estimated to be 60–65 kDa. Although 22 ns represents a lower limit to the actual overall correlation time of the complex, these results encouraged the acquisition of triple-resonance NMR experiments for the assignment and structure determination of OprH in DHPC micelles.

Assignment of nitrogen, hydrogen, Cα/H9251, Cβ/H9252, and CO resonances for 2H-,13C-,15N-labeled OprH in DHPC micelles was achieved by using three pairs of TROSY-based triple resonance NMR experiments to establish through-bond connectivity: HNCA, HN(CO)CA, HN(CA)CB, HN(COCA)CB, HNCO, and HN(CA)CO. By this method, 89% of the nitrogen and hydrogen resonances, 94% of the Cα resonances, 91% of the Cβ resonances, and 93% of the CO resonances were assigned. The remaining resonances were not assignable due to excessive line broadening or ambiguity. Complete backbone chemical shift assignments were obtained for 156 of the 180 residues (excluding the C-terminal His6 tag and lead methionine) in the OprH-His6 construct. Only partial assignments could be obtained for 13 residues, which included the 2 prolines, and 11 residues remained completely unassigned. Local secondary structures determined from the secondary Cα and Cβ chemical shifts (29) are shown in Fig. 4. Eight distinct regions

FIGURE 3. 15N-1H TROSY spectrum of 2H-,13C-,15N-labeled OprH in DHPC micelles collected at 800 MHz and 45 °C. The refolded protein sample was exchanged into 25 mM NaPO4 at pH 6.1, 50 mM KCl, 0.05% NaN3, and 5% D2O before being concentrated to ~1.0 mM for NMR experiments. Assignments determined as described under “Results” are shown. For some residues, all found in the micelle-solvent interfacial region of the protein, a second set of weaker peaks could be assigned. These residues are denoted with an apostrophe.
of large negative values characteristic of β-strands are separated by regions of values around zero, indicative of turns or random coils.

The NOEs to generate structural distance constraints were acquired through 15N-1H-1H NOESY-TROSY and 15N-1H-15N HSQC-NOESY-HSQC experiments. Redundancy from the acquisition of both spectra reduced any ambiguities resulting from overlapping peaks, and the assignments of many NOEs could be verified through two pairs of cross-peaks. A total of 221 and 285 peaks were assigned and integrated in the 15N-1H-1H NOESY-TROSY and 15N-15N-1H HSQC-NOESY-HSQC spectra, respectively. Distance constraints were preferentially calculated from NOEs derived from the 15N-1H-1H NOESY-TROSY spectrum unless there was severe peak overlap, in which case the 15N-15N-1H HSQC-NOESY-HSQC spectrum was utilized. The pattern of long-range NOEs in both spectra was characteristic of antiparallel β-sheet secondary structure. Many of the residue pairs responsible for the stronger long-range NOEs were identified as participating in hydrogen bonds through 2H/1H exchange experiments (supplemental Fig. 5). Based upon these 2H/1H exchange results and the pattern of strong NOEs, a total of 134 hydrogen bond...
constraints were generated (Fig. 5). Only two of these constraints between Phe-50–Gly-53 were short- or medium-range.

The structure of OprH in DHPC micelles was calculated from 199 NOE-derived distance constraints, 188 chemical shift-derived backbone dihedral angle constraints (23), and 134 hydrogen bond constraints. The overall features of the lowest energy conformer from the 20 lowest energy NMR structure ensembles (Fig. 6) revealed an eight-stranded antiparallel β-barrel (β-strands β1–β8) connected by four extracellular loops and three smaller periplasmic turns. A common feature of β-barrel OM proteins is the presence of two girdles of aromatic residues located at the β-barrel rim that partition into the membrane-water interface and define the membrane-embedded boundaries of the protein (30, 31). As shown in Fig. 6C, quite a large number of aromatic residues are located at and define the outer interfacial rim of OprH. However, these residues are more sparse around the periplasmic β-barrel rim. Additionally, the side chains of the aromatic residues Tyr-55 and Tyr-80 are more centrally located in the nonpolar membrane interior.

The well defined β-barrel region of OprH had an average β-strand length of 11 residues and an average pairwise backbone root mean square deviation (r.m.s.d.) of 0.85 ± 0.20 Å in the NMR ensemble (Table 1). We also calculated the structures without hydrogen bond constraints, in which case the same overall fold, but with an average pairwise backbone r.m.s.d. of 0.97 ± 0.15 Å, was obtained. The shear number (32) of the β-barrel was 10, and the tilt angle with respect to the membrane normal (31) was 43°. Nearly all of the residues that remained unassigned are located in the extracellular loops of OprH except for the C-terminal His6 tag and the lead methionine (Fig. 5). The small number of long-range NOEs in the extracellular loop regions resulted in a decreased structural definition in these parts of the structure and an overall backbone r.m.s.d. of 6.55 ± 1.40 Å when the loops are included. The periplasmic turns were well defined and, when considered with the β-barrel, produced a backbone r.m.s.d. of 1.03 ± 0.19 Å. Overall, these results indicate that the precision of the structural ensemble of the OprH global fold was comparable with that of other OM β-barrel proteins determined by solution NMR spectroscopy.

Dynamics of OprH in DHPC Micelles—To determine whether the lack of structural information obtained from loop residues was due to increased backbone dynamics in these regions or due to other issues, we measured longitudinal ($T_1$) and transverse ($T_2$) relaxation times along with $^{1}H$-$^{15}N$ heteronuclear NOEs at 800 MHz and 45 °C. These data are sensi-
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TABLE 1

| NMR and refinement statistics for OprH structures in DHPC micelles |
|---------------------------------------------------------------|
| Results were calculated among the 20 lowest energy CNS conformers of the structure of OprH in DHPC micelles. |

| NMR distance and dihedral angle constraints | Structure calculation | Unique HN-HN NOE distances | 199 |
|---------------------------------------------|-----------------------|-----------------------------|-----|
|                                             | Sequential            | 93                          |     |
|                                             | Medium range          | 11                          |     |
|                                             | Long range            | 95                          |     |
|                                             | Intermolecular         | 0                           |     |
| Hydrogen bond constraints                    | 134                    |                             |     |
| Dihedral angle constraints                   | 188                    |                             |     |

| Violations | Distance constraints (Å) | 0.017 ± 0.001 |
|           | Dihedral angle constraints (°) | 0.148 ± 0.020 |
|           | Maximum distance constraint (>0.2 Å) | 0 |
|           | Maximum dihedral angle (>2.0°) | 0 |
|           | Deviations from ideal geometry | 
| Bond lengths (Å) | 0.001 ± 0.00004 |
| Bond angles (°) | 0.2780 ± 0.0020 |
| Improperss   | 0.1106 ± 0.0063 |

| Ramachandran map analysis & | Most favored regions (%) | 66.0 |
|-----------------------------|--------------------------|-----|
| Additionally allowed regions (%) | 29.3 |
| Generously allowed regions (%) | 3.4 |
| Disallowed regions (%) | 1.3 |

| Ensemble r.m.s.d. | Mean global backbone r.m.s. deviation (Å) | 0.85 ± 0.20 |
|-------------------|------------------------------------------|-------------|
|                   | β-Sheet residues                          | 1.03 ± 0.19 |
|                   | β-Sheet and turn residues                 | 6.55 ± 1.40 |
|                   | All residues                             | 2.07 ± 0.26 |
|                   | β-Sheet and turn residues                 | 2.80 ± 0.23 |
|                   | All residues                             | 7.24 ± 1.29 |

| a Calculated using PROCHECK-NMR. |
| b β-Sheet residues are defined as 3–12, 41–50, 53–60, 73–85, 92–104, 117–130, 136–147, and 170–179 from the mean of the 20 conformers. |

Table 1 highlights the NMR and refinement statistics for OprH structures in DHPC micelles. The results were calculated among the 20 lowest energy CNS conformers of the structure of OprH in DHPC micelles, with specific focus on the structural constraints and deviations from ideal geometry.

Different Regions of OprH Interact with LPS—To further determine which specific residues of OprH interact with LPS, purified LPS from *P. aeruginosa* PAO1 was added directly to refolded [3H]-[15N]-labeled OprH in DHPC micelles in the presence of EDTA, which resulted in an NMR sample that contained a DHPC:LPS:OprH molar ratio of 150:10:1. Based upon the results from the trypsin protection of OprH by LPS as shown in Fig. 2, this molar ratio should saturate binding of LPS to OprH. The [15N]-H TROSY spectrum acquired from this sample was still well dispersed. From the overlay of the spectrum onto the spectrum of OprH in DHPC, it was apparent that the addition of LPS resulted in a significant number of chemical shift perturbations (supplemental Fig. 6A). The [15N]-H TROSY spectrum of OprH in DHPC:LPS was then assigned, utilizing the assignments of OprH in DHPC-only micelles with an additional single-triple-resonance HNCA experiment with the LPS-containing sample. The resulting chemical shift perturbations could then be mapped onto the lowest energy conformer of the OprH structure ensemble (Fig. 8A). The most significant chemical shift perturbations occurred for residues at the base of the extracellular loop 1 (Gly-11, Gly-12, Gly-40, Tyr-41, and Trp-42). These residues constitute a localized region of negative charge on the surface of the barrel (Fig. 8B). Large chemical shift perturbations were also observed in negatively charged residues located in turns 2 and 3 (Asn-90 and Glu-130). The backbone chemical shift perturbations for residues located in the β-barrel region were mostly minor with the exceptions of the central tyrosine residues Tyr-55 and Tyr-80 (Fig. 8, A and B). Relatively small chemical shift perturbations were observed for residues located in the more positively charged extracellular loops, namely: Arg-25 and Leu-27 in loop 1; Asn-69 in loop 2; Gly-110 and Phe-111 in loop 3; and Lys-159 and Leu-163 in loop 4. By direct comparison of a [15N]-H NOESY-TROSY spectrum acquired for [3H],[13C],[15N]-labeled OprH in DHPC:LPS mixed micelles to that of OprH in DHPC only micelles, ~80% of the long-range distance constraints could be confirmed through identical NOEs, whereas the remaining NOEs were unobservable, most likely due to line broadening. Thus, the addition of LPS to the micelle did not result in changes to the global fold of the protein. When MgCl<sub>2</sub> was added to OprH in DHPC:LPS, the TROSY spectrum was indistinguishable from the spectrum acquired in the presence of EDTA except for a few minor chemical shift differences in three unassigned cross-peaks (supplemental Fig. 6B). The addition of 6 mM 1,2-dimyristoyl-sn-glycero-3-phosphocholine, which has an acyl chain length of 14-carbons, to OprH in DHPC micelles resulted in only minor chemical shift perturbations in a limited number of [15N]-TROSY resonances, indicating that the perturbations resulting from the addition of LPS were not solely a consequence of the increased acyl chain length of LPS relative to DHPC (supplemental Fig. 6, C and D).

Based upon the NMR and protease protection assay results for OprH in DHPC:LPS mixed micelles, four OprH deletion mutants were made. Each mutant contained a deletion for a different extracellular loop of OprH to further examine whether eliminating any one of these loops could abolish LPS protection of trypsin digestion. The specific constructs were: OprHAΔ17–38 (ΔL1), OprHAΔ65–72 (ΔL2), OprHAΔ108–114 (ΔL3), and OprHAΔ150–162 (ΔL4). Each OprH mutant in DHPC:LPS mixed micelles was treated with trypsin in the absence or presence of MgCl<sub>2</sub> and boiled in SDS-PAGE loading buffer, and the resulting cleavage products were analyzed by SDS-PAGE (Fig. 9). LPS provided protection from trypsin digestion for all the OprH deletion mutants, indicating that no single loop deletion was sufficient to abolish OprH-LPS binding in the absence of Mg<sup>2+</sup>.

The susceptibility of each specific cleavage site in loops 2, 3, and 4 was assessed by qualitatively evaluating the apparent
molecular weight differences of the protected bands for each respective deletion mutant. The smallest observed band in the absence and presence of MgCl₂ for the OprH (ΔL3) mutation had an apparent molecular mass of ~13 kDa (Fig. 9A, lanes 5 and 11). As shown in supplemental Fig. 7, this cleavage product was also observed for the OprH (ΔL3) mutant in DHPC micelles and could only result from the complete protection of the loop 2 residues Lys-70 and Arg-72 from trypsin digestion in both DHPC micelles and DHPC:LPS mixed micelles. The disappearance of cleavage products <13 kDa also indicated that the concentration-dependent increase of trypsin protection observed for full-length OprH in the absence of Mg²⁺, as shown in Fig. 2, B and C, was a result of increasing LPS protection of residues Lys-109 and Lys-112 in loop 3. Two protected bands were observed for all the deletion mutants in the absence of MgCl₂ except for OprH (ΔL4) (Fig. 9A, lane6). Removal of the trypsin cleavage site at residue Lys-159 in extracellular loop 4 by the OprH (ΔL4) mutation resulted in only one protected band, indicating that residue Lys-159 in the full-length OprH remained partially accessible to trypsin cleavage in the presence of LPS. The diagram in Fig. 9B shows the obtained cleavage products for the wild type and for each deletion mutant. Based

FIGURE 7. Backbone dynamics of OprH. A–C, the longitudinal relaxation times (A), transverse relaxation times (B), and [¹H]-¹⁵N heteronuclear NOEs (C) of OprH in DHPC micelles determined at 800 MHz and 45 °C are plotted as a function of the amino acid sequence. Blue bars in the T₁ and T₂ plots are the upper limits of the standard deviations. Blue bars in the NOE plot represent the upper limits of the standard errors. The secondary structure pattern observed in the solution structure is shown on the bottom.
upon these results, the susceptibility of the extracellular loops of OprH to trypsin cleavage in the presence of LPS can be defined as: loop 1/loop 4/loop 3/loop 2.

DISCUSSION

The newly determined structure of OprH consists of an eight-stranded antiparallel β-barrel with four extracellular loops and three periplasmic turns (Fig. 6). To the best of our knowledge, this is only the fifth polytopic integral membrane protein structure that has been solved by NMR before crystal structures were known. The others are PagP from E. coli (28) and the mitochondrial anion channel VDAC-1 (35), for both of which a crystal structure was solved later, E. coli diacylglycerol kinase (36), and the murine mitochondrial uncoupling protein 2 (37). The topology and secondary structure of OprH refolded into DHPC micelles resemble a model that was previously generated by hydropathy analysis and subsequently validated by insertion and deletion mutagenesis on OprH that was overexpressed in P. aeruginosa OMs (13). Most β-strands terminate within ±2 residues from the previously predicted model, except for β2 and β3, which extend for an additional 5 and 3 residues in the extracellular direction, respectively. Six of the eight β-strands (β2–β7) also extend farther on the periplasmic side, resulting in tighter periplasmic turns in the structure than in the predicted model. Because these differences are relatively small, interpretations of previous biological results requiring only knowledge of the topology of OprH remain valid.

Like in several other outer membrane proteins whose structures have been solved by NMR, the secondary Ca and Cβ chemical shifts, the longitudinal and transverse relaxation times, and the [1H]-15N heteronuclear NOE values of OprH in DHPC micelles show that the extracellular loops of OprH are disordered and extended. Interestingly, many residues located at the barrel-loop interface have NOE values comparable with those observed for the β-barrel residues, indicating restricted

FIGURE 8. Effect of LPS on the amide backbone resonances of OprH in DHPC:LPS mixed micelles. A, chemical shift perturbations between 15N-1H TROSY spectra of 2H, 13C, 15N-labeled OprH in DHPC micelles (150:1, DHPC:OprH molar ratio) and DHPC:LPS mixed micelles (150:10:1, DHPC:LPS:OprH molar ratio) in the presence of 5 mM EDTA were determined. These differences are shown as compound chemical shift changes (Δδ_comp = (ΔδHN + (ΔδNN/6.5)}^{1/2})(48) mapped color-coded onto the lowest energy structure of OprH in DHPC micelles. B, electrostatic surface potential plots of the lowest energy structure of OprH in DHPC micelles generated using the charge-smoothing algorithm in PyMOL (49).
motions in these regions. When considering the shorter $T_2$ values indicative of intermediate conformational exchange observed in these residues (Fig. 7), it is clear that the structure has unique slow dynamic properties in the interfacial regions at both rims of the barrel. Similar observations have been made for other outer membrane $\beta$-barrel proteins, including OmpA and KpOmpA (38–40). Not unlike many other $\beta$-barrel membrane proteins, OprH has a girdle of aromatic residues at the outer barrel rim, but fewer such residues at the inner periplasmic rim. Instead, OprH features two adjacent prominent tyrosines near the center of the lipid bilayer membrane (Fig. 6).

Previous studies provided only circumstantial indirect evidence that LPS might be interacting with OprH in Pseudomonas outer membranes, an interaction that has been hypothesized to contribute to the antibiotic resistance of these bacteria under low Mg$^{2+}$ conditions (11, 12). In this work, we utilized in vivo and in vitro assays to demonstrate a direct interaction between the P. aeruginosa OM protein OprH and LPS. These results are further bolstered and refined by NMR interaction studies using the newly determined structure.

The observation that significantly more LPS was co-immunoprecipitated by OprH from P. aeruginosa membrane lysates
that were prepared from cultures grown in the presence of low Mg$^{2+}$ relative to cultures grown in high Mg$^{2+}$ suggests that other genes that influence the affinity of OprH for LPS are up-regulated in a Mg$^{2+}$-dependent fashion. Although OprH expression in the PAO1ΔoprH mutant strain used in the immunoprecipitation assays was abolished, the upstream promoter of the oprH-phoP-phoQ operon was not altered. Therefore, PAO1ΔoprH retained endogenous expression of the phoP-phoQ locus. This is important because phoPQ controls the pmrAB two-component regulatory system, which in turn regulates structural alterations in LPS that increase resistance to polymyxin B (41–43). The addition of aminoarabinose to the 1’- and 4’-phosphates of the LPS lipid A domain, as well as the addition of palmitate to lipid A, resulting from P. aeruginosa PAO1 growth in low Mg$^{2+}$ medium, has been previously documented (44). Therefore, the increase in the amount of LPS co-immunoprecipitated from the low Mg$^{2+}$ lysates by OprH indicates that such LPS structural alterations could lead to an increase in the affinity of OprH for LPS.

The trypsin digestion results with purified OprH that was refolded into DHPC micelles in vitro indicate that purified and reconstituted OprH retains its native ability to bind unmodified LPS. Note that the LPS used in these assays was purified from P. aeruginosa PAO1 grown in Mg$^{2+}$-sufficient medium, and therefore, the LPS lipid A domain did not contain the additional aminoarabinose and palmitate moieties found under low Mg$^{2+}$ conditions. Additionally, the complete reversal of LPS protection from trypsin digestion upon the addition of Mg$^{2+}$ provides the first direct evidence that OprH acts as a surrogate for Mg$^{2+}$ by cross-linking LPS, thereby tightening the outer membrane during divalent cation deficiency.

The notion that LPS binds directly to OprH is supported by our NMR chemical shift perturbation experiments. The resonances of numerous residues were significantly shifted by LPS, but not by another more generic long-chain lipid (1,2-dimyristoyl-sn-glycero-3-phosphocholine) that was added to the mixed protein-detergent micelles. Because we were able to derive the structure of OprH in membrane-mimetic DHPC micelles, these chemical shift perturbations could be mapped to specific regions on this structure to identify LPS interaction sites. Multiple LPS interaction sites in different regions of the structure were identified by these experiments (Fig. 8). Interestingly, most of these sites corresponded to the more negatively charged areas on the surface of the protein, most notably at the base of loops 1 and 3 and in the periplasmic turns 2 and 3. Conspicuous LPS-specific chemical shift perturbations were also found for the 2 unusual centrally located tyrosine residues. Chemical or physical reasons for these observed interactions are presently not well understood, but will be the target of future interaction studies.

Detailed molecular interactions of LPS have been previously reported with another OM protein, namely the ferric hydroxamate uptake A (FhuA) protein from E. coli whose crystal structure was solved in complex with LPS (45). In this case, binding is directed by numerous strong electrostatic interactions between basic amino acid residues of FhuA with acidic phosphate moieties located in the lipid A and inner core moieties of LPS. Overall, the binding of FhuA to LPS encompasses a large surface area including at least four β-strands and two extracellular loops. In addition to the interactions between OprH and LPS described above, our NMR results suggest a similar mechanism of LPS binding to OprH. Although only weak chemical shift perturbations were observed in these regions of the protein, the lysines and arginines of the extracellular loops 2 and 3 may form hydrogen bonds with the phosphate moieties of the lipid A domain of LPS. This binding mode would place the glucosamine backbone of lipid A in close proximity to the base of extracellular loop 1, where the largest chemical shift perturbations were observed. Although this interpretation is also supported by the protection of the OprH extracellular loops 2 and 3 from trypsin digestion by LPS, it is not unlikely that OprH may interact with more than one LPS molecule as its function is to replace Mg$^{2+}$ and stabilize the P. aeruginosa OM. It is also important to keep in mind that our studies so far only recorded perturbations at the polypeptide backbone level and that specific interactions with side chains are beyond the reach of the present experiments. Therefore, a more definitive molecular model for interactions between OprH and LPS will have to await the use of specific side-chain isotope labeling and possibly the use of different LPS precursors in future efforts.

In conclusion, we have presented evidence for the interaction between OprH and LPS both in native P. aeruginosa outer membranes and in a model membrane system using both biochemical and biophysical techniques. Beyond determining the structure of OprH, our study also demonstrates that solution NMR is a powerful tool to examine interactions of integral membrane proteins with specific lipids in a fully solvated lipidic environment, which cannot be easily done by crystallography. Overall, the results of this study provide new insight into the structure and role of OprH in P. aeruginosa outer membranes while offering new evidence for protein-lipid interactions that likely contribute to antibiotic resistance during P. aeruginosa infections.

Acknowledgments—We acknowledge the W. M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia. We thank Ming-Tao Pai, Ph.D. for initial work on this project.

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