Structural basis for solute transport, nucleotide regulation, and immunological recognition of *Neisseria meningitidis* PorB

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Edited by Douglas C Rees, Caltech/HHMI, Pasadena, CA, and approved March 4, 2010 (received for review October 21, 2009)

PorB is the second most prevalent outer membrane protein in *Neisseria meningitidis*. PorB is required for neisserial pathogenesis and can elicit a Toll-like receptor mediated host immune response. Here, the x-ray crystal structure of PorB has been determined to 2.3 Å resolution. Structural analysis and cocryrstallization studies identify three putative solute translocation pathways through the channel pore: One pathway transports anions nonselectively, one transports cations nonselectively, and one facilitates the specific uptake of sugars. During infection, PorB likely binds host mitochondrial ATP, and cocryrstallization with the ATP analog AMP–PNP suggests that binding of nucleotides regulates these translocation pathways both by partial occlusion of the pore and by restricting the motion of a putative voltage gating loop. PorB is located on the surface of *N. meningitidis* and can be recognized by receptors of the host innate immune system. Features of PorB suggest that Toll-like receptor mediated recognition outer membrane proteins may be initiated with a nonspecific electrostatic attraction.

*Neisseria* are Gram-negative bacteria with only two pathogenic species: *Neisseria meningitidis* and *Neisseria gonorrhoeae*, which cause bacterial meningitis and gonorrhoea, respectively (1). Similar to other Gram-negative bacteria, solute translocation across the outer membrane is essential for neisserial survival. In *N. meningitidis*, PorB is the second most prevalent outer membrane protein (OMP), and has previously been characterized as a voltage-gated nonselective porin (2) that translocates smaller sugars faster than larger sugars (3).

Both *N. meningitidis* and *N. gonorrhoeae* PorB [60% identical; (4, 5)] are believed to contribute to disease progression (6). During infection, PorB targets the mitochondria (7, 8) where it likely inserts into the inner membrane (9). Once inserted, these porins noncovalently bind ATP (10). Functionally, the presence of ATP reduces the single-channel conductance and increases the open probability (9). In addition, PorB directly interacts with the host mitochondrial voltage-dependent anion channel (VDAC) (7). Importantly, the insertion of PorB into mitochondrial membranes is known to influence apoptosis (7–9, 11–13). Because both the mitochondrial membrane potential and VDAC are important for apoptosis (14), PorB may influence the initiation of apoptosis at multiple checkpoints.

In addition to the contribution of PorB to disease progression, it may also impair treatment. In *N. gonorrhoeae*, the *PenB* antibiotic resistance mutations map to the PorB (15, 16). In conjunction with mutations in the PorA porin and the MtrR transcription factor, these *PenB* mutations confer intermediate resistance to penicillin and tetracycline.

Recognition of *N. meningitidis* and other pathogenic bacteria by host toll-like receptors (TLRs) is the first step in the activation of the inflammatory responses of the innate immune system (17, 18). TLRs recognize various molecular components of pathogens but do not undergo maturation to improve affinity. TLR1, TLR2, TLR6, and MD2 have each been suggested to be involved in the recognition of a broad range of OMPs (19–23). Specifically, TLR2 has been shown to recognize PorB, likely within the physiological context of a TLR1/2 heterodimer (22). The basis for the recognition of OMPs by TLRs is difficult to envision because OMPs vary in sequence, structure, diameter, and conductance.

Understanding TLR recognition of OMPs is also important because their misactivation may underlie a broad variety of autoimmune diseases. Specifically, TLR2 polymorphisms and increased signaling are associated with inflammatory bowel disease and Crohn’s Disease (24, 25), suggesting that inappropriate reactivity to the symbiotic flora of the gut contributes to inflammation in these disorders. Identifying how TLRs appropriately recognize pathogens may shed light on these disease processes.

Here, the structure of the *N. meningitidis* PorB has been determined to 2.3 Å resolution alone and in complex with sucrose, galactose, and AMP–PNP. These structures suggest that PorB utilizes different faces of the pore to facilitate selective and nonselective substrate transport, and that nucleotide binding regulates translocation along these pathways. Further, analysis of the structure suggests a possible mechanism for the recognition of OMPs by TLRs of the innate immune system during the host response to infection.

Results

Structural Overview. The structure of PorB cloned from a patient isolate of *N. meningitidis* (26) was determined by the method of multiple isomorphous replacement with anomalous scattering (ref. 27 and Fig. 1). Calculation of interpretable electron density maps (Fig. S1) required multiple crystal form averaging between a P63 and an R32 crystal form (*SI Text* and Tables S1 and S2). Both crystal forms contain a monomer in each asymmetric unit with the trimer forming around a crystallographic 3-fold symmetry axis (Fig. 1 A and B). Each PorB monomer forms a 16-stranded β-barrel with short turns connecting the strands on the periplasmic side of the channel, and long, interstrand loops on the extracellular side of the channel. In particular, extracellul-

www.pnas.org/cgi/doi/10.1073/pnas.09121151107

PNAS | April 13, 2010 | vol. 107 | no. 15 | 6811–6816

Author contributions: M.T. and T.M.I. designed research; M.T., C.M.N., and T.M.I. performed research; M.T., C.M.N., and T.M.I. analyzed data; and M.T. and T.M.I. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The coordinates and structure factors for *N. meningitidis* PorB have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank with accession codes 3A2R (native), 3A2S (sucrose), 3A2T (galactose), and 3A2U (AMP–PNP).

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This article contains supporting information online at www.pnas.org/cgi/content/full/0912115107/DCSupplemental.
lar loop 3 (L3), protrudes into the pore and forms an α-helix, which constricts the pore to 8 Å by 10 Å at its narrowest point. This topology (Fig. S2) is similar to that observed for other OMPs, with sequence insertions and variability in the region exposed to the host immune system (ref. 28; for a comparison between PorB and other Gram-negative porins, see SI Text and Fig. S3). The sequence of PorB from the patient isolate used for this study (26) contains 2 mutations that differ from the reported protein sequence (5), both of which are located in the extracellular loops (Fig. S2). Whereas this clinical isolate was associated with an epidemic outbreak of meningitis, it is not known if these particular mutations in PorB affect virulence.

Sugar Binding and Selectivity. To identify the molecular determinants for solute translocation by PorB, the electrostatic surface potential was calculated (Fig. 1 B and C). This analysis revealed an unusual distribution of surface charges. On the extracellular side of the channel, the funnel approaching the pore is strongly electronegative (Fig. 1B), whereas on the periplasmic side of the channel, the funnel is strongly electropositive (Fig. 1C). Although PorB has previously been characterized as a nonselective channel, these electrostatics differ from those of other nonselective porins of known structure, which have consistent, intermediate charge (29, 30). Because pore funnels with strongly opposing charges are observed in some substrate-specific channels (31, 32), sugar binding was evaluated.

Previous sugar uptake assays showed that PorB transports small sugars more quickly than larger sugars (3). To identify whether PorB could translocate other sugars, uptake assays were performed in osmotically active sugars (3). Galactose and arabinose are similar in size to glucose and also showed fast transport through PorB, whereas sucrose and maltose displayed much slower transport rates.

Cocrystallization of PorB with sugar substrates revealed a specific binding site for both galactose (Fig. 2A and B) and sucrose (Fig. 2D) at the same location (Fig. 2E) within the positively charged funnel. A positively charged cage formed by the side chains of arginine and lysine residues surrounds the sugar-binding site, and a similar sugar-binding motif can be identified in other sugar-binding porins (Fig. S4).

Putative Nonselective Translocation Pathways. Nonselective substrate translocation pathways are challenging to identify by x-ray crystallography because the solutes are unlikely to make specific interactions with the channel. However, the location of bound heavy atoms in the derivative datasets and previous studies of antibiotic resistance mutations (15, 16) hint at the location of a cation-selective pathway in PorB. The Lu\(^{3+}\) and Er\(^{3+}\) derivative datasets (SI Text and Table S2) identify an electronegative location approaching the pore funnel where cations may be attracted prior to translocation. Both Lu\(^{3+}\) and Er\(^{3+}\) are localized near the upper part of the helix in loop L3 between the side chain carboxylates of Asp117 and Glu202 and the backbone carbonyl of Gly122 (Fig. S5 A and B). However, neither is within direct coordination distance of the protein (SI Text and Fig. S5 C and D), suggesting that water molecules, which cannot be observed at the resolution of either heavy atom dataset, mediate their association. A cation-binding site mediated by water molecules would allow for plasticity in the coordination sphere, translating to promiscuity of solute transport.

Located immediately below the cation-binding sites are Gly103 and Asp104, which confer intermediate resistance to penicillin and tetracycline when mutated in the N. gonorrhoeae PorB (refs. 15 and 16 and Figs. S2 and S5 E and F). A decrease in conductance and open probability associated with these mutations (16) is consistent with these side chains lining a substrate translocation pathway.

Although PorB translocates cations, it has been electrophysiologically characterized as a weakly anion selective (33), suggesting that an anion pathway must be available. Examination of the electrostatic surface representation (Fig. 1 B and C) identifies a putative pathway on the pore face nearest the crystallographic 3-fold axis that is lined with positively charged residues. A similar feature has been identified in the Escherichia coli OmpF (34, 35), the Delftica acidovorans Omp32 (36, 37), and the E. coli OmpC (38), which all share structural similarity to PorB in the transmembrane β-barrel region of the protein (SI Text and Fig. S3 A–C). Lining the putative anion translocation pathway are four conserved Lysines and Arginines (Fig. S3D). Site-directed mutagenesis (39–41), crystallographic analyses (36, 42), and molecular dynamics simulations (35, 36) suggest that the Lysine/Arginine motif indeed acts as an anion translocation pathway. In PorB, the putative cation and anion translocation pathways do not overlap with the sugar-binding site or each other, suggesting that selective sugar transport and promiscuous cation and anion translocation through PorB are independent.

Regulation of Conductance by Purine Nucleotide Triphosphates. An intriguing characteristic of PorB is the regulation of channel conductance by purine nucleotide triphosphates (pNTPs; see ref. 10). Physiologically, this likely occurs during infection when the channel is inserted into the host mitochondrial membrane. Cocrystallization of PorB with the ATP analog AMP–PNP resulted in clear electron density within the pore (Fig. 3A). This binding site is surrounded by the side chains of Lys9, Lys42, Arg77, Asn96, Lys100, and Arg130 and is adjacent to L3 near the pore constriction (Fig. 3B). An exhaustive search of pNTP-binding proteins in the Protein Data Bank used a combination of automated and manual approaches, but did not identify any known pNTP-binding motifs that had a similar three-dimensional arrangement of stabilizing contacts between protein and pNTP. This suggests that PorB has
of 5 mM of either pNTP had little effect on sugar uptake rates of PorB. Addition and sugars also overlap. As a result, the effect of ATP and AMP anion translocation pathway, the binding site for the AMP (Fig. 3 D) cation and anion translocation pathways to different extents (9) and increases channel open probability (9). The AMP–PNP binding site overlaps with the sugar-binding site and putative (10) and increases channel open probability (9). The AMP–PNP binding site overlaps with the sugar-binding site and putative cation and anion translocation pathways to different extents (Fig. 3D). Whereas greatest physical overlap is with the putative anion translocation pathway, the binding site for the AMP–PNP and sugars also overlap. As a result, the effect of ATP and AMP–PNP on sugar transport through PorB was investigated. Addition of 5 mM of either pNTP had little effect on sugar uptake rates into PorB-containing liposomes (Fig. S6).

**Interaction Between PorB and TLR2.** The purified ectodomain of human TLR2 was evaluated for direct complex formation with PorB (SI Text and Fig. S7). Coinjection of sTLR2 and PorB onto a size exclusion column resulted in a monodisperse species with an approximate molecular weight of 405 kD, consistent with a physiological trimer (43) and TLRs likely signal as dimers (22, 44), the 2:6 stoichiometry is consistent with known oligomerization states. An increase in NaCl concentration from 100 mM to 300 mM during the experiment decreased complex formation (Fig. S7), consistent with electrostatics contributing to the TLR–PorB interaction.

**Discussion**

**Multiple Translocation Pathways in Porins.** Historically, porins have been classified as either broadly nonspecific with large conductances, or substrate-selective with small conductances. However, studies on the *E. coli* OmpF (35) and the *Aquifax aeolicus* AQ_1862 (45) and the *D. acidovorans* Omp32 (36) suggest that porins may in fact be multiply selective with sophisticated mechanisms for substrate selection.

**Sugar Translocation.** Previous reports of the cocrystal structures of the sucrose-specific porin ScrY (46) and maltoporin LamB (47) both suggested that aromatic residues are important for sugar transport. However, the sugar-binding site identified in PorB lacks aromatic residues. Instead, a positively charged cage consisting of Arg and Lys residues (RK cage) appears to be important for binding sugar molecules in PorB (Fig. S4 A and B). This RK cage forms a rectangle that is approximately 11–12 Å on one side and approximately 6–8 Å on the other side. A similar RK cage is observed in both ScrY (Fig. S4 C and D and ref. 46) and LamB (Fig. S4 E and F and ref. 47). A notable difference in sugar binding between ScrY, LamB, and PorB, is that in ScrY and LamB, the sugar is coordinated by side chains from both faces of the funnel leading to the pore. In PorB, the sugars bind asymmetrically to one face of the funnel (Fig. 2 and Fig. S4), allowing the other faces to remain available for the translocation of other substrates (Fig. 3D).

**Mechanisms of Gating.** The molecular determinants of voltage gating in β-barrel channels is not clear, although a number of mechanisms have been proposed. Most OMPs contain a region of sequence that folds within the center of the β-barrel and forms an integral part of the pore (30, 48). Studies of the pH-regulated
Fig. 3. Binding of AMP–PNP and regulation of channel conductance by pNTPs. (A) Binding of AMP–PNP. $|F_o| - |F_i|$ omit maps contoured at 3σ calculated after the omission of AMP–PNP (Black). (B) Specific side chain contacts to AMP–PNP. (C) Comparison of the electrostatic surface potential calculated with APBS (61) of PorB with no ligands bound (PDBID:3A2R, Left) to the electrostatic surface potential with AMP–PNP bound (PDBID:3A2U, Right). Positive potential is colored blue, and negative potential is colored red. Potentials are contoured from $-20\, kT/e$ to $-20\, kT/e$. The diameter of pore is $8 \times 10\, \text{Å}$ without AMP–PNP, and narrows to $4 \times 5\, \text{Å}$ when AMP–PNP is bound. The view is from the extracellular side of the channel. (D) Binding sites and putative transport pathways through PorB. The binding sites for sugars are indicated with magenta (galactose) and cyan (sucrose) circles, the binding site for AMP–PNP is indicated with a black rectangle, the binding site for cations is indicated with orange circle, and the binding site for antibiotics is shown with a red triangle. A putative cation translocation pathway lined by negatively charged amino acids is highlighted with orange shading, whereas the putative anion translocation pathways lined with positively charged side chains is highlighted with blue shading.

OmpG from *E. coli* (49, 50) and the VDAC of mitochondria (51, 52) suggest that in these cases, the folded domain within the pore is important for gating. In Gram-negative porins, including PorB, this folded domain is located on interstrand L3, which forms an α-helix that may contribute to gating. In the structure of PorB, L3 constricts but does not close the pore. The exposed side chains of L3 are nearly exclusively negatively charged, initially suggesting that the application of an electric field across the membrane could induce L3 to undergo a movement that could physically obstruct the pore (53).

In PorB, both voltage and pNTP ligands can modulate channel conductance. In this way, PorB is functionally similar to VDAC (10), which can be regulated by both voltage and the binding of the ligand NADH (54, 55). The cocrystal structure of PorB with AMP–PNP shows that this pNTP analog affects both the electrostatics of the funnel approaching the pore and the pore diameter. Taken together, this likely affects the identity of solutes that can traverse the pore directly because it may alter size cutoff for solutes and may shift the selectivity to more positively charged solutes. Supporting a direct effect of pNTP on solute translocation, previous single-channel bilayer recordings of PorB performed in the presence and absence of ATP show that ATP indeed decreases single-channel conductance (9, 10).

The location of the AMP–PNP observed in this structure suggests that in addition to the direct modulation of the characteristics of the channel pore, pNTPs may alter voltage gating. AMP–PNP binds adjacent to the hinge of L3, possibly restricting its motion upon voltage change. The restriction of the motion of L3 could prevent its obstruction of the pore when voltage changes across the membrane thus maintaining the open probability high over a wide range of voltages. Supporting this hypothesis, recordings of PorB channels in bilayers showed that ATP impeded voltage gating by preventing closure of the pores at large membrane potentials and maintaining high open probability over the entire voltage range tested (9).

At first glance, the observed lack of effect of ATP on the rates of sugar transport in PorB-reconstituted liposomes (Fig. S6) appears to contrast with the finding that showed that ATP leads to a smaller single-channel conduction (9, 10). However, the liposomal transport assays are a function of both unitary-transport rate and open probability. Because ATP appears to decrease the unitary rate but at the same time appears to increase the open probability of these channels, the two effects may cancel each other out in the macroliposomal transport rate measurement. It is also possible that the transport assay is not sufficiently sensitive to measure ATP-induced changes.

A Model for TLR-Mediated Recognition of OMPs. The ability of TLRs to recognize cognate ligand without affinity maturation requires a recognition mode that uses nonspecific interactions to fundamental features of the pathogen. As an example, in the recognition of dsRNA, TLRs recognize elements of the RNA backbone, an element that is not organism specific and cannot undergo evolution to escape human defense mechanisms (56). The first identified ligands for TLR2 contained covalent lipopolysaccharides (LPS) modification, suggesting that TLR2 recognized this unique modification. However, not all TLR2 ligands are LPS-modified, and PorB falls into this category. In the absence of a specific, unique feature on the protein, the proposal of a recognition mechanism of the TLR1/2 heterodimer for OMPs is conceptually challenging, because proteins widely vary in their molecular properties. However, the analysis of the crystal structures of PorB and the TLR1/2 heterodimer (57) allowed the development of a model for the initial recognition of OMPs by TLR1/2. PorB and other Gram-negative porins contain a ring of positively charged residues on the extracellular side of the protein. This evolutionarily conserved ring of positive charges may be important for guiding the directional insertion of this protein into the membrane, and is proposed to interact with the negatively charged lipopolysaccharides to stabilize the porin within the bacterial outer membrane (37, 58). An electrostatic analysis of the TLR1/2 heterodimer reveals that both ectodomains, which mediate recognition, are predominantly negatively charged (Fig. 4). This negatively charged surface could be attracted to the ring of positive charges from PorB. Supporting the role of electrostatics in complex affinity, increasing the salt concentration decreased complex formation (Fig. S7). Because this external ring of positive charges is an evolutionarily conserved feature of OMPs, a contributing
factor to TLR-mediated recognition of OMPs may be nonspecific electrostatic attraction. The identification of more specific details of this interaction requires an experimental costructure of the TRL1/2-PorB complex.

TLR-mediated innate immunity is activated by OMPs from pathogens but not by the OMPs of commensal bacteria, which also contain a ring of positive charges on the extracellular side of the protein. In addition, N. meningitidis itself is rarely pathogenic and found in the commensal flora in a significant percentage of the population (59). Under these conditions, it does not activate innate immunity. Thus, following initial binding of receptor to OMP, additional factors must be required for innate immune activation.

Materials and Methods

Protein Expression and Purification. The gene encoding PorB was amplified from a clinical isolate of Neisseria meningitidis (serotype W135 (26)), and cloned into the pET21b expression vector with a stop codon included prior to the plasmid-encoded C-terminal hexahistidine (His6) tag. The protein was expressed and purified as described (27) (SI Text).

Crystal Structure Determination. R32 and P66 crystals of PorB were grown by mixing 1.0 μl protein solution with an equal volume of reservoir solution containing 100 mM MES buffer pH 6.0–7.0, 50 mM CsCl, 28–32% (w/v) Jefamine M-600. Native data were collected using wavelengths and beamlines listed in Table S1. The structure was determined using isomorphous and anomalous contributions for three heavy atom derivatives: Lu3+, Er3+, and WO42− (SI Text and Table S2). For cocrystallization with substrates, preformed crystals were soaked in reservoir solution containing 5 mM AMP-PNP, 1% galactose, or 5% sucrose for 3–5 d (Table S1).

Liposome Uptake Assays. Osmotically active liposomes were prepared according to refs. 16 and 60 using E. coli total lipids (Avanti). Cholesterol-solubilized lipids were dried, washed with pentane, and resuspended in water. A mixture of 0.1–1 μg PorB per mg of lipid was sonicated until translucent, dried in a cooled speedvac, and stored in a dessicator in the dark overnight. Liposomes were then resuspended in 5 mM Dextran T-40, 5 mM Tris, pH 8 and left at room temperature for 30 min. For the uptake assays, 10–15 μL of the proteoliposomes were added to 0.5 mL of various sugar solutions (stachylose hydrate from Stachys tuberifera, D-glucose, sucrose, D-galactose, D-maltose, L-arabino–ose) in 5 mM Tris, pH 8. Liposome swelling was measured in triplicate by monitoring changes in optical density at 400 nm at 1 s intervals for 180 s. Readings were normalized to the maximal optical density for each time course. The sugar concentration isoosmotic to the intraplastomic milieu was determined for each preparation by identifying the concentration of the impermeant sugar stachylose that did not elicit liposomal swelling upon dilution. Transport of each sugar through each liposome preparation was assayed at the predetermined isoosmotic concentration for that preparation.

Acknowledgments. We thank Yi Wei Tang for the clinical isolate of N. meningitidis; Scott Meier for purified Cu2+ protein; César Luna-Chávez and Sanjay Mishra for assistance with MultiAngle Laser Light Scattering experiments; Jessica Vey, Timothy Panosian, and Thomas Tomasiak for assistance with the data collection; and Chuck Sanders, Yi Wei Tang, Paola Massari, Anitra Prenger, and Tasia Pyburn for critical reading of the manuscript.

The growth of N. meningitidis was supported by National Institutes of Health Grants GM081816 and GM079419 (T.M.) and GM077560 (C.M.N.), American Heart Association Grant 0630168Z (C.M.N.), and The Uehara Memorial Foundation and Bundesminister für Bildung und Forschung (M.T.). Portions of this research were carried out at beamlines 9-2 and 11-1 at Stanford Synchrotron Radiation Light Source, a national user facility operated by Stanford University on behalf of the US Department of Energy, Office of Basic Energy Sciences. Use of the Life Sciences Collaborative Access Team ID21G at Advanced Photon Source was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract DE-AC02-06CH11357. Beamline 12.3.1 at the Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, US Department of Energy under Contract DE-AC02-05CH11231. The Cornell High Energy Synchrotron Source (CHESS) F2 stations are supported by the National Science Foundation and the National Institutes of Health National Institute of General Medical Sciences via NSF Grant DMR-0225180, and the MacCHESS resource is supported by the National Institutes of Health National Center for Research Resources Grant RR-01646.


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