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Crystallographic analysis of *Neisseria meningitidis* PorB extracellular loops potentially implicated in TLR2 recognition

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Abstract

Among all *Neisseriae* species, *N. meningitidis* and *N. gonorrhoeae* are the only human pathogens, causative agents of bacterial meningitis and gonorrhoea, respectively. PorB, a pan-*Neisseriae* trimeric porin that mediates diffusive transport of essential molecules across the bacterial outer membrane, is also known to activate host innate immunity via Toll-like receptor 2 (TLR2)-mediated signaling. The molecular mechanism of PorB binding to TLR2 is not known, but it has been hypothesized that electrostatic interactions contribute to ligand/receptor binding. Strain-specific sequence variability in the surface-exposed loops of PorB which are potentially implicated in TLR2 binding, may explain the difference in TLR2-mediated cell activation *in vitro* by PorB homologs from the commensal *N. lactamica* and the pathogen *N. meningitidis*. Here, we report a comparative structural analysis of PorB from *N. meningitidis* serogroup B strain 8765 (63% sequence homology with PorB from *N. meningitidis* serogroup W135) and a mutant in which amino acid substitutions in the extracellular loop 7 lead to significantly reduced TLR2-dependent activity *in vitro*. We observe that this mutation both alters the loop conformation and causes dramatic changes of electrostatic surface charge, both of which may affect TLR2 recognition and signalling.

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Accession Numbers

The Protein Data Bank accession numbers for the atomic coordinates and structure factors for PorB^{WT} and PorB^{DDE/AKR} are 3W14 and 3W15 respectively.

The authors declare no conflict of interest.

Keywords

outer membrane protein; *Neisseriae*; crystal structure; Toll-like receptor

1. Neisserial PorB structure and functions

Neisseria meningitidis is a Gram-negative opportunistic human pathogen carried by approximately 10–15% of the adult population (Cartwright et al., 1987; Yazdankhah and Caugant, 2004). Upon colonization of the nasopharyngeal epithelium, host cell invasion and meningococci dissemination via the bloodstream may occur, leading to meningitis and septicaemia. Among thirteen existing *N. meningitidis* serogroups, the majority of meningococcal disease worldwide is caused by A, B, C, X, Y and W135 types, categorized according to their capsular polysaccharides. Despite recent advances in meningococcal vaccine developments, *N. meningitidis* serogroup B strains still pose a threat worldwide. Nasopharyngeal colonization by *N. lactamica* is also frequent, particularly in infants and young children, but systemic infections are very rare and this organism is considered a commensal (Gold et al., 1978). Within the *Neisseria* family, *N. gonorrhoeae* is also a human pathogen and the causative agent of gonorrhoea (Virji, 2009).

Porins, major Gram-negative bacteria outer membrane proteins, mediate diffusive transport of essential solutes across the membrane (Nikaido, 2003). *N. meningitidis* expresses two porins, PorA and PorB (either class 2 or class 3), while *N. lactamica* only expresses PorB. PorB has a 16-stranded β -barrel structure with eight surface-exposed loops (L1 – L8) and corresponding shorter periplasmic turns. While the β -barrel regions share a high level of sequence homology among the different strains, amino acid sequence variability characterizes the surface-exposed loops (van der Ley et al., 1991; Derrick et al., 1999; Bennett et al., 2008). The variable loop regions (VR) 1 to 4, located in L1, L5, L6 and L7, are used for the classification of *N. meningitidis* clonal complexes. *N. meningitidis* PorB (strain W135) and *N. gonorrhoeae* PorB_{IA} (strain N242) have been recently crystallized (Tanabe et al., 2010; Zeth et al., 2013), confirming the predicted PorB topology model.

Besides having conventional porin functions, PorB influences host cell invasion, intracellular bacteria survival, immune evasion and even onset of disease. For example, PorB-induced host cell actin nucleation and reorganization during host cell infection possibly influences bacterial invasiveness (Wen et al., 2000), and PorB translocation into the host cell mitochondrial membrane modulates host cell apoptosis (Massari et al., 2000; Binnicker et al., 2004; Follows et al., 2009; Massari et al., 2010; Jiang et al., 2011; Kozjak-Pavlovic et al., 2009).

One of the best-characterized PorB functions *in vitro* and *in vivo* is induction of host cell signalling via Toll-like receptor 2 (TLR2) and TLR1 (Massari et al., 2002; Massari et al., 2006; Toussi et al., 2012), resulting in its immune adjuvant properties (Wetzler et al., 1996; Mackinnon et al., 1999; Burke et al., 2007; Liu et al., 2008; Chiavolini et al., 2008). TLRs recognize pathogen-associated molecular patterns (PAMPs) and their structural features have been extensively studied (Botos et al., 2011). PAMP recognition by the extracellular domain of TLR2 and TLR1 induces dimerization and rearrangement of their cytoplasmic domains, recruitment of the adaptor protein MyD88 and downstream activation of NF- κ B, MAPKs and AP-1 signalling pathways (Medzhitov et al., 1998).

Although the TLR2-dependent activity of PorB is well-characterized, studies on the molecular details of PorB/TLR2 interaction are scarce. A possible model proposes electrostatic interaction of negatively charged residues on the TLR2 ectodomain and positively charged residues in PorB surface-exposed regions (Tanabe et al., 2010). PorB

directly binds to TLR2 *in vitro* and to cell surface-bound TLR2 (Massari et al., 2006;Liu et al., 2010;Toussi et al., 2012). However, PorB from different *Neisseriae* strains have different apparent affinity for TLR2 and induce variable levels of cell activation, possibly via different intracellular signalling pathways. Strain-specific *porB* gene sequence variability within these regions may influence the modality of interaction with TLR2 and subsequent cell activation, as shown by studies using *N. meningitidis*/*N. lactamica* hybrid PorB loop mutants (Massari et al., 2006;Liu et al., 2010;Toussi et al., 2012).

As a step towards the characterization of PorB/TLR2 interaction, we report the crystal structure and molecular differences between PorB from *N. meningitidis* serogroup B strain 8765 (PorB^{WT}, which shares 63% sequence identity with PorB from serogroup W135 (PorB^{W135}) (Tanabe and Iverson, 2009) and the PorB^{DDE255-262AKR} mutant (PorB^{DDE/AKR}), in which mutation of three residues in loop 7 for corresponding residues from the sequence of *N. lactamica* PorB (PorB^{Nl}) causes a significant reduction in TLR2-dependent host cell responses *in vitro* (Toussi et al., 2012). Comparison of PorB^{WT} and PorB^{DDE/AKR} crystal structures shows an altered L7 loop conformation, modification in charge distribution and electrostatic surface differences that likely influence PorB/TLR2 interaction and subsequent cell activation levels. Understanding the mechanism of PorB recognition by TLR2 at a molecular level will provide invaluable information for studies of its functions *in vitro* and *in vivo*.

2. Recombinant PorB expression and purification

The plasmid pNVK15, encoding PorB from *N. meningitidis* serogroup B strain 8765 (B:15:P1,3) and generation of PorB loop mutants have been previously described (Qi et al., 1994;Toussi et al., 2012). All PorB molecules were expressed as inclusion bodies in *E. coli* BL21(DE3) and purified as also previously described (Tanabe and Iverson, 2009), with minor modifications. Briefly, prior to a single-step size exclusion chromatography (HiPrep Sephacryl S-100 16/60), samples were incubated for 4 days at 4 °C in 20 mM Tris-HCl pH 7.5, 200 mM NaCl and 0.1% lauryldimethylamine N-oxide (LDAO) (Buffer A) to enhance refolding and dialyzed against Buffer A (30:1 ratio) using a 10 kDa molecular weight cut-off dialysis tubing.

3. Crystallization, data collection, structure determination and molecular dynamics simulation

PorB^{WT} crystals grew in 0.1 M MES pH 6.5 – 7.0, 50 mM CsCl and 29 – 35% (v/v) Jeffamine M-600 within 4 months and the L7 mutant PorB^{DDE/AKR} crystals grew in 65 mM HEPES pH 7.5 and 1.1 M tri-sodium citrate in 2 weeks using previously described methods (Kattner et al., 2013). PorB^{DDE/AKR} and PorB^{WT} crystals were flash-cooled in liquid nitrogen in 1.5 M tri-sodium citrate with 20% Jeffamine M-600 or with 35% of Jeffamine M-600 as cryoprotectant, respectively. PorB^{WT} diffraction data were collected at the Swiss Light Source (SLS) PX-II (X10SA) and for PorB^{DDE/AKR} at the PX-III (X06DA) beamline at wavelengths of 1.00 Å. Datasets were processed using the HKL2000 suite (Otwinowski and Minor, 1997) and structures were solved by the molecular replacement (MR) method with Phaser (McCoy, 2007) using PDB ID: 3VZT as search model (Tanabe et al., 2010). Model building and refinement were performed using COOT (Emsley and Cowtan, 2004) and REFMAC5 (Murshudov et al., 1997) in CCP4 suite (Winn et al., 2011) and structure quality was analyzed by PROCHECK (Laskowski et al., 1993). Figures were generated with Pymol (DeLano, 2002) and sequence alignment performed by ClustalW (Thompson et al., 1994). The predicted structural models of selected PorB mutants were calculated by RosettaBackrub (Smith and Kortemme, 2008).

All-atom molecular dynamics simulations of PorB in bilayers were performed in GROMACS4.6 (Pronk et al., 2013). We used a combination of the Amber99sb-ildn force field (Lindorff-Larsen et al., 2010) for trimeric PorB, together with the virtual site model for hydrogen atoms (Feenstra et al., 1999), the Berger model for lipids (Cordomi et al., 2012) and the SPC/E water model (Berendsen et al., 1987), as employed previously for PorB (Kutzner et al., 2011). The trimers were inserted into fully hydrated and equilibrated POPC bilayers (Cordomi et al., 2012) by using the GROMACS utility *g_membed* (Wolf et al., 2010), equilibrated further for 25 ns under gradual release of position restraints on lipid head groups and the protein, and then freely simulated in the NpT-ensemble for 300 ns (integration time-step: 4 fs) at 1 bar and T=300K by using Berendsen pressure (Berendsen et al., 1984) and velocity-rescale temperature coupling (Bussi et al., 2007).

4. Structure of PorB from *N. meningitidis* strain 8765

Crystals of PorB^{WT} were grown in Jeffamine M-600, similar to PorB^{W135} (Tanabe and Iverson, 2009). Mutants of PorB^{WT} in L4, L5 and L7 were recently described (Toussi et al., 2012). The L7 mutant, PorB^{DDE/AKR}, which induces significantly lower TLR2-dependent cell activation *in vitro* than PorB^{WT}, was instead crystallized using a salt-based precipitant, a condition that was not successful for PorB^{WT}. Crystals of PorB^{WT} and PorB^{DDE/AKR} diffracted to 3.3 and 2.4 Å, respectively (Table 1).

Initial MR modeling of the PorB^{WT} and PorB^{DDE/AKR} crystal structures based on the coordinates of PorB^{W135} (PDB ID: 3VZT) presented some difficulties in refinement of the loop regions, since these molecules share only 63% of overall amino acid sequence identity and rms deviation values for the C α atoms of 0.78 Å for (269 residues). The model rebuilding was facilitated by the structure of PorB_{1A} from *N. gonorrhoeae* strain AN242 (PorB^{Ng}) (PDB ID: 4AUI) (Zeth et al., 2013). This porin has a 77% sequence identity to PorB^{WT} and rms deviation values for the C α atoms of 0.99 Å for (271 residues), allowing a significant improvement of loop tracing. Data collection and refinement statistics are summarized in Table 1. PorB^{WT} has a final R_{work}/R_{free} value of 25.3 / 29.3% for data refined to 3.3 Å and PorB^{DDE/AKR} a final R_{work}/R_{free} value of 22.7% / 25.5% for data refined to 2.4 Å.

Like other conventional porins, PorB^{WT} and PorB^{DDE/AKR} are organized around a 16-stranded β -barrel structure with eight long, extracellular surface-exposed loops and seven short periplasmic turns. The β -sheet regions are highly conserved among all Neisserial PorB molecules but the loop structures are rather different (Fig. 1). PorB^{WT} and PorB^{DDE/AKR} crystal structures form trimers around a crystallographic 3-fold symmetry axis but are packed in two different arrangements (Supplementary Fig. S1). PorB^{WT} shows a possible back-to-back crystal contact site between adjacent molecules on the periplasmic side, and via L1 - L1 and L6 - L6 on the extracellular side. PorB^{DDE/AKR} crystal contacts appear only mediated by L1 in a trimer and by L6 in the adjacent trimer and, possibly, on the interface region between L6 and L7. No crystal contact on the periplasmic side is observed.

5. Central pore region

In most Gram-negative diffusive porins, L3 is extended and protrudes into the pore channel forming a constriction area. Regulation of channel properties by this loop is well studied (Nikaido, 2003). No major structural change is observed in L3 between PorB^{WT}, PorB^{DDE/AKR}, PorB^{W135} and even PorB^{Ng}. The L3 in PorB^{WT} and PorB^{DDE/AKR} also protrudes towards the inner region of the central pore, narrowing it to approximately 8 × 12 Å.

Interestingly, two large extra densities are observed in the central pore and do not correspond to any area of the polypeptide chain of PorB. It is possible that these are citrate molecules deriving from the crystallization buffer, coordinated by the positively charged residues K63, R76, K99, and R125 for citrate 1 and K41 for citrate 2 (Supplementary Fig.S2). Citrates are negatively charged in solution and, here, localize in the middle of the previously identified anion transport pathway (Kattner et al., 2013), consistent with the existence of separate cation/anion transport pathways.

6. Extracellular Loops Structure Comparison

A high structural variability is observed in the surface-exposed loops of PorB^{WT}, PorB^{DDE/AKR}, PorB^{W135}, PorB^{NI} and PorB^{Ng} (Fig. 1). For example, the amino acid sequence of L4 and L5 in both PorB^{WT} and PorB^{NI} is significantly shorter than that of PorB^{W135}. In PorB^{WT}, the sharp-turn region of L5 is not well-ordered and the electron density of residues 186–188 was not observed; by contrast, the remaining loops are reasonably well-ordered and the C α trace was clearly visible (Fig. 2).

Overall, subtle structural differences are evident in the crystal structure of PorB^{WT} and PorB^{DDE/AKR}. The comparison of loop rms deviation values is shown in Table 2. L7 is well-structured in both PorB^{WT} and PorB^{DDE/AKR} and its electron density is clear. The mutation in L7 loop might cause a large structural change between these PorBs (Fig. 2A–C). No major direct contribution of L7 to the macromolecular assembly of PorB^{WT} crystal packing is observed (Supplementary Fig. S1). Although there is no strong contribution of L7 in the crystal packing of PorB^{DDE/AKR}, residues at the interface region between L6 and L7, i.e. L220, D224, S226 in L6, might mediate crystal contacts through T27 and V29 in the β -strand 2 and possibly surface-located residues, such as K225, L257 in L7 might contact Q22 and D23 in L1 from another trimer.

Analysis of L7 of PorB^{DDE/AKR} reveals a tilt of approximately 60° and the C α of K260 is shifted of 15 Å towards the inner part of the pore, as compared to PorB^{WT}. The distance between E186 in L5 and K260 in L7 is close enough for a weak ionic interaction that may stabilize L7 conformation and contribute to its inward bending in PorB^{DDE/AKR} (Fig. 2D). When inward bending of L7 is not observed, such as in PorB^{WT}, L7 cannot interact with L5 and has a relatively vertical shape.

The electron density of residues 186–188 in L5 of PorB^{WT} was not observed clearly. The same loop in PorB^{DDE/AKR} is bent inward of approximately 1.8–2 Å (the distance comparison of C α of Q185 and I190 between two structures (Fig. 2E)), more than in PorB^{WT}, likely affecting the potential L5 and L7 interactions. Computational modeling of PorB^{WT} and PorB^{DDE/AKR} also supports different loop structures and loop bending caused by the DDE to AKR mutation (Supplementary Fig. S3). Thus, although the crystal contacts might affect L7 bending, structure predictions recapitulate our analysis of PorB^{DDE/AKR} loop structure.

The B-factor of PorB^{WT} and PorB^{DDE/AKR}, plotted in Fig. 3A, shows that all extracellular loops, including L7, the periplasmic turns and also L3 behave similarly. This observation suggests that our model of the structural differences of L7 in PorB^{WT} and PorB^{DDE/AKR} is not depicting an accidental snap-shot due to loop bending by crystal packing, but more likely results from the mutations.

To investigate the conformation of L7 in more detail, molecular dynamics simulations were performed on PorB^{WT} and PorB^{DDE/AKR} trimers immersed in POPC bilayers (Fig. 3B). The simulations support the conclusion that the overall conformation of L7, i.e. whether it folds upward or inward, is determined by the mutation, as the general conformation seen for L7 of

PorB^{WT} and PorB^{DDE/AKR} in the crystal is stable, despite the usual fluctuation of a loop region, for each subunit in 300-ns simulations (Fig. 3B, Supplementary Fig. S4). This result suggests that the crystal loop conformations are not determined by crystal packing, and that L7 in PorB^{DDE/AKR} is likely to consistently protrude into the pore region.

7. Electrostatic charge distribution in PorB

Based on the hypothesis that PorB binding to TLR2 may be through electrostatic interactions, it is likely that charged residues in L7 play a role in this interaction. Analysis of the net porin charges reveals an overall negative charge of -2 ($+32/-34$) for PorB^{WT}. Mutation of DDE to AKR in L7 of PorB^{WT} leads to a positive charge of $+3$ ($+34/-31$) in PorB^{DDE/AKR}. Positive charges of $+10$ ($+44/-34$) and $+5$ ($+37/-32$) are also observed for PorB^{W135} and PorB^{Ng}, respectively. The loop area charge distribution (excluding L2 and L3, which are unlikely to be exposed in the surface area) is even more striking, -3 ($+9/-12$) for PorB^{WT}, $+2$ ($+11/-9$) for PorB^{DDE/AKR}, due to L7 residues mutation, and $+6$ ($+18/-12$) for PorB^{W135}. The charge distribution in L7 is -3 ($+1/-4$) for PorB^{WT} while it is $+2$ ($+3/-1$) for PorB^{DDE/AKR} and PorB^{W135}. The calculation of electrostatic surface charge reveals that the L7 vertical positioning in PorB^{WT} exposes a strong negatively-charged area, while its inward bending in PorB^{DDE/AKR} leads to a neutrally-charged region in a potential TLR2-binding area (Fig. 4).

8. Hypothetical TLR2/PorB interaction mechanism

Differences in TLR2-dependent activity and apparent binding affinity have been reported for *N. meningitidis* PorB and *N. lactamica* PorB (Massari et al., 2006; Liu et al., 2010; Toussi et al., 2012). A major question is whether these differences may be due to sequence variations of the loops that hypothetically interact with TLR2. We observed several differences in the crystal structure of PorB^{WT} and PorB^{DDE/AKR}.

First, from a conformational perspective, the vertical positioning of L7 in PorB^{WT} places it against the membrane side, while its inward bending in PorB^{DDE/AKR} suggests a reduced exposure to the protein's extracellular side. This could result in a potential different TLR2 recognition modality. By modeling the conformation of another characterized PorB^{WT} loop mutant (Toussi et al., 2012), PorB^{GEG} (a Gly-Glu-Gly insertion between H181 and H182 in L5), loop elongation and a similar bending towards the inside of the pore is predicted (Supplementary Fig. S3C). PorB^{GEG} has a modest TLR2-dependent activity *in vitro*, although not as low as PorB^{DDE/AKR}. The *in vitro* activity of another PorB^{WT} mutant, in which a leucine in L4 was substituted by a proline to alter the loop structure, is almost comparable to that of PorB^{WT}, despite the different loop conformation (Supplementary Fig. S3C).

Furthermore, the activity of a loop 5 and 7 double mutant, (PorB^{GEG-DDE/AKR}) is comparable to that of the single L5 mutant, PorB^{GEG} (not shown). While this is consistently lower than that of PorB^{WT} but higher than that of PorB^{DDE/AKR}, this mutant also retains the ability to inhibit PorB^{WT} binding to TLR2 on the cell surface, similar to both PorB^{GEG} and PorB^{DDE/AKR} (Toussi et al., 2012).

These results suggest that TLR2 binding and subsequent induction of signaling may be affected by concerted conformational changes due to both individual loop conformation as well as reduced exposure of the molecule's extracellular side area that can directly contact TLR2. However, the vertical exposure of L7 appears required for an effective interaction with TLR2.

Second, from an electrostatic surface charge perspective, we have observed more dramatic changes around L7 region between PorB^{WT} and PorB^{DDE/AKR} (Fig. 4). PorB^{WT} has a net charge of −3 for the extracellular loop regions while that of PorB^{DDE/AKR} is shifted to +2. Interestingly, PorB^{W135} also has a net loop region positive charge value of +6, which may reflect its lower activity compared to PorB^{WT} (D. Toussi and P. Massari, unpublished results), similar to the *in vitro* activity of PorB^{DDE/AKR}. The structure of L7 of PorB^{W135} resembles that of PorB^{WT} more than that of PorB^{DDE/AKR} (rms deviation comparison of Ca atoms of L7 between PorB^{W135} and PorB^{WT} is 1.9 Å, while between PorB^{W135} and PorB^{DDE/AKR} is 3.9 Å). The charge of L7 is −3 for PorB^{WT} and +2 for PorB^{DDE/AKR} and PorB^{W135}, suggesting that, if charge differences contribute to TLR binding, dramatic changes of the electrostatic surface charge environment in the vicinity of L7 region may affect TLR recruitment. Interestingly, since even in the presence of high salt concentrations PorB/TLR complexes can still be formed (Tanabe et al., 2010), charged residues appear to play an important role, but other structural restrictions, especially in L7, could contribute to TLR2 binding, explaining the relatively modest decrease of TLR-dependent activity of L4, L5 and L5/7 mutants. Thus, although initially we proposed that positive-charged residues are involved in PorB interaction with TLR2 (Tanabe et al., 2010), from the present study it appears that negative charges also play a relevant role, especially in the L7 region. The precise contribution of each individual loop in the interaction with TLR2 is not trivial and still remains to be determined. Our ongoing studies to define PorB/TLR2 complex formation will further explore the mechanism of their molecular interaction and provide novel insights into a general mechanism of outer membrane protein recognition by TLRs.

We have shown that PorB^{WT} and PorB^{NI} induce different levels of TLR2-dependent IL-8 and RANTES secretion *in vitro* (Toussi et al., 2012), an inflammatory cytokines secretion trend that is similar to that reported for the clinical consequences of meningococcal infections and disease severity (Carrol et al., 2005; Moller et al., 2005). It is possible that *Neisseriae* strains that express PorB encoding specific residues in L1–L8 (with the exception of L2 and L3), result in different PorB/TLR2 specific interaction and subsequent host cell responses at the site of colonization that may influence pathogenicity. For example, some natural mutants of PorB variable regions correlate with clinical isolates with different invasive potential (Urwin et al., 2004; Law et al., 2004; Yang et al., 2009). In conclusion, our analysis of the crystal structure of *N. meningitidis* 8765 PorB and of a L7 mutant, PorB^{DDE/AKR}, supports that changes in both electrostatic surface charge and associated conformation difference of the L7 region could be important factors for TLR2 recognition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Structure comparison and sequence alignment of PorB molecules with known crystal structure. **A)** The overlay of Ca trace cartoon model is shown from membrane side and extracellular side. PorB^{WT}, gray. PorB^{DDE/AKR}, cyan. PorB^{W135}, green. PorB^{Ng}, pale pink. The predicted membrane-embedded area is light-blue shadowed. **B)** Sequences of PorB^{WT}, PorB^{W135}, PorB^{Ng} and of PorB^{NI} (from *N. lactamica* strain Y92-1009) performed with ClustalW. β-sheet regions, light blue columns. Connecting loops, black lines. Residues that are 100% conserved, blue letters. Mutated residues in L7 for PorB^{DDE/AKR}, red.

Fig. 2. Superimposition of the C α trace models of the extracellular portion of L7 of PorB^{WT} (gray) and PorB^{DDE/AKR} (cyan) (**A**). The 2|F_o| - |F_c| map of L7 mutated region of PorB^{WT} (light gray) (**B**) and PorB^{DDE/AKR} (light cyan) (**C**) with electron density contoured at +1.0 σ . **D**) Close-up view of potentially interacting residues in L5 (E186) and in L7 (K260) of PorB^{DDE/AKR} (light cyan) or in L7 (D260) of PorB^{WT} (light gray). **E**) Close-up view of L5 region of PorB^{WT} (light gray) and PorB^{DDE/AKR} (light cyan).

Fig. 3.

(A) B-factor superimposition of the cartoon models of PorB^{WT} and PorB^{DDE/AKR}. Dark blue denotes areas of low B-factor. Higher B-factor areas are colored in green, orange, yellow and red, respectively. The average of B-factor of each loop is shown in table. (B) Exemplary loop conformations of L7 and L5 (red, orange) representing the largest deviations from the crystal conformation (green) observed in our 300-ns simulations of PorB^{WT} (left) and PorB^{DDE/AKR} (right). The general configuration of L7 is stable, folding consistently upward in PorB^{WT} and into the barrel in PorB^{DDE/AKR}. L5 shows slightly greater conformational deviations than L7.

Fig. 4. Comparison of PorB electrostatic surfaces. Electrostatic surface potential comparison were calculated using Adaptive Poisson–Boltzmann Solver (APBS) (Baker et al., 2001). Regions with positive potential are shown in blue and with negative potential in red. Potentials are contoured from -20 kT/e to 20 kT/e. Top panel, view from the extracellular side. Bottom panel, view from the periplasmic side showing the electrostatic distribution of the channel interior.

Table 1

Data collection and Refinement Statistics

	Wild-type (PorB ^{WT})	PorB ^{DDE/AKR}
<i>Data collection</i>		
Beamline	SLS PX-II	SLS PX-III
Wavelength	1.00 Å	1.00 Å
Space group	R32 (H32)	R32 (H32)
Unit cell constants <i>a</i> , <i>b</i> , <i>c</i> (Å)	148.3, 148.3, 104.0	143.4, 143.4, 178.4
Resolution (Å)	50-3.30 (3.37-3.30)*	50-2.40 (2.45-2.40)
No. of total reflections	17921	171041
No. of unique reflections	6628	27700
R _{sym}	0.099 (0.280)	0.084 (0.500)
I/σ (I)	9.55(3.54)	17.4 (1.55)
Redundancy	2.9 (2.8)	6.7 (3.9)
Completeness (%)	92.8 (76.9)	92.2 (73.0)
<i>Refinement</i>		
Resolution	3.30	2.40
# R _{work} (%)	25.4	22.7
* R _{free} (%)	29.3	25.6
No. of atoms	2380	2501
Protein	2380	2393
Citrate	-	26
Water	-	82
B-factor (protein)	56.6	50.6
B-factors (citrate)	-	93.9
B-factors (water)	-	51.3
<i>Rms deviations</i>		
Bond length (Å)	0.012	0.016
Bond angles (deg)	1.93	1.63
Ramachandran plot		
Most favored regions	90.1%	90.6%
Additional allowed regions	9.9%	9.4%
Disallowed regions	0%	0%

R_{work} is the *R*-factor = $\sum ||F_O| - |F_C|| / \sum |F_O|$, where *F_O* and *F_C* are the observed and calculated structure factors, respectively.

* R_{free} is the same as R_{cryst} calculated on 10 % of the reflections in PorB^{WT} and 7 % of the reflections in PorB^{DDE/AKR} that were omitted during the refinement process.

* Values in parentheses are for the highest resolution shell

Table 2Rms deviation of surface exposed loops between PorB^{WT} and PorB^{DDE/AKR}

	rmsd value
L1	1.19
L2	0.47
L3	0.49
L4	0.45
L5	0.69
L6	0.77
L7	4.41
L8	1.61
Overall	0.60