Structure
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Structural and Dynamical Insights into the Opening Mechanism of *P. aeruginosa* OprM Channel

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SUMMARY

Originally described in bacteria, drug transporters are now recognized as major determinants in antibiotics resistance. For Gram-negative bacteria, the reversible assembly consisting of an inner membrane protein responsible for the active transport, a periplasmic protein, and an exit outer membrane channel achieves transport. The opening of the outer membrane protein OprM from Pseudomonas aeruginosa was modeled through normal mode analysis starting from a new X-ray structure solved at 2.4 Å resolution in $P2_12_12_1$ space group. The three monomers are not linked by internal crystallographic symmetries highlighting the possible functional differences. This structure is closed at both ends, but modeling allowed for an opening that is not reduced to the classically proposed "iris-like mechanism."

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium that causes opportunistic infections in immunocompromised patients and exhibits natural and acquired resistance to diverse antibiotics (Nakae, 1997). The analysis of the genome sequence of this bacterium has revealed the existence of 12 potential resistance-nodulation-cell division (RND) efflux systems (Stover et al., 2000), 11 of which have been biochemically described (Aendekerk et al., 2002; Aires et al., 1999; Chuanchuen et al., 2002; Hassan et al., 1999; Kohler et al., 1997; Kumar and Schweizer, 2005; Mima et al., 2005, 2007; Poole et al., 1993, 1996). Among these, the MexAB-OprM efflux system is the only one being constitutively produced in wild-type strains (Nakajima et al., 2002). The operon is controlled by a transcription regulator MexR. The first gene of each operon encodes MexA (MW 38,592 Da/monomer) a periplasmic membrane fusion protein (MFP); the second encodes MexB (MW 112,790 Da/monomer), a cytoplasmic membrane RND protein presumed to act as a drug-proton antiporter; and the third gene encodes OprM (MW 50,867 Da/monomer), an outer membrane channel-forming factor (OMF). All three proteins are believed to form a complex channel that goes across both membranes and allows the extrusion of drugs directly into the extracellular medium (Mokhonov et al., 2004; Tikhonova et al., 2002; Zgurskaya and Nikaido, 2000), bypassing the periplasmic space. Whereas OprM can function with different RND transporter/MFP complexes from *P. aeruginosa*, other outer membrane proteins such as OprN are more discriminative and do not interact with MexAB (Maseda et al., 2000) although sharing 48% amino acid sequence homology with OprM. The most similar homolog of the MexAB-OprM efflux system is the extensively studied AcrAB-TolC system (Okusu et al., 1996) found in *Escherichia coli*, which is also involved in multiple antimicrobial resistance through an energy-dependent efflux mechanism (Fralick, 1996). One other homologous porin from *vibrio cholerae* is the VceC from VceABC system, for which only the VceC crystal structure has been solved (Federici et al., 2005).

The sequence identity between MexA-MexB-OprM and AcrA-AcrB-TolC are 55%, 69%, and 19%, respectively. The X-ray structures of the three components of the *E. coli* pump have already been determined. Four structures have been published for the homotrimer TolC (Koronakis et al., 2000; Higgins et al., 2004; Bavro et al., 2008), one for AcrA (Mikolosko et al., 2006) and more than 20 for AcrB (Murakami et al., 2002, 2006; Yu et al., 2003, 2005; Su et al., 2006; Seeger et al., 2006; Sennhauser et al., 2007; Das et al., 2007; Törnroth-Horsefield et al., 2007; Drew et al., 2008; Veesler et al., 2008). The AcrB protein also forms a homotrimer that corresponds to the functional unit (Murakami et al., 2004). The first structures of AcrB were determined in space groups in which the three monomers were linked by internal three-fold crystallographic symmetry. Those constrained structures could not help explaining the mechanism of the pump. Only when the nonsymmetrical structure of Murakami and Seeger was published in 2006 (Murakami et al., 2006) could they visualize the differences among the three monomers, leading to the peristaltic pump mechanism concept.

Here, we report a new OprM X-ray structure that, in contrast to the previously published one by Akama et al. (2004), does not present internal crystallographic symmetry, thereby allowing us to build each protomer independently and highlight subtle differences between each protomer within the trimer. The relatively low thermal factor of this OprM structure allowed us to observe most of the first hydration layer, inside and outside the channel.

As observed for TolC, the crystal structure of OprM shows a closed channel within the protein. Mutagenesis, coupled to functional assays based on electrophysiology measurements, have allowed depicting key residues for maintaining the closed state (Andersen et al., 2002), but at present, no study has ever been able to give strong evidence demonstrating the periplasmic opening mechanism. We assumed that in silico molecular modeling would offer the possibility of exploring the dynamics of the residues implicated in the opening. In this context, normal mode analysis (NMA) already proved to be a powerful and relevant tool: trajectories along low-frequency modes give insights into large conformational changes occurring within large proteins. Using our OprM high-resolution X-ray structure coordinates, we have indeed successfully identified normal modes liable to describe the periplasmic opening of the protein. Importantly, in contrast to other in silico studies, for which mutants were required to simulate open structures, the opening mechanism presented here is obtained for the wild-type forms of OprM and TolC. Hence, besides a high-resolution structure of OprM, this study yields new insights in the OprM protein gating and enlightens subtle differences that exist between TolC and OprM.

RESULTS AND DISCUSSION

Structure of the Trimer of OprM Not Constrained by a Three-Fold Symmetry Axis and Comparison with TolC

Here we present the crystal structure of OprM homotrimer in $P2_12_12_1$ space group without three-fold crystal symmetry at a resolution of 2.4 Å. Phases were solved by molecular replacement with the previously published OprM structure (Akama et al., 2004; Protein Data Bank [PDB] code 1WP1), using a poly-alanine model in order to reveal potential differences among the three monomers. Probably due to high flexibility, the electron density of each monomer is missing the 13 residues at the C terminus.

Structural comparison of OprM with TolC (Koronakis et al., 2000) (Figure 1) shows similar folding (root-mean-square deviation [rmsd] of 1.6 Å calculated from C-α) in spite of a relatively low sequence identity (19%) (Figure 2). A common gene duplication concerning this porin family is suggested by a structural repeat of a two-stranded β sheet and a coiled-coil α-helical pair, within one monomer. In OprM, the N-terminal half (residues 61 to 239, Figure 1 numbering) and the C-terminal half (residues 273 to 447) can be aligned with a sequence identity of 22% and superposed with an rmsd calculated using C-α of 3.3 Å. The α-helical pair H7/H8 of the C-terminal half presents an additional kink at A375 in OprM as opposed to its counterpart, the α-helical pair H3/H4 of the N-terminal half. This kink only appears as a more pronounced curvature change in H7 of TolC. This difference may suggest a functional implication as a hinge region during the opening mechanism of the channel, as will be discussed along normal modes analysis.

The topology of the equatorial domains of OprM and TolC is quite different and does not show a duplication of a structural pattern. Common features are two α helices (H1 and H5 in Figure 1) and a large loop between H4 and H5 implicated in the recruitment of the MFP partner (Yamanaka et al., 2007). It also has to be noticed that the two helices, η2-OprM and H9-TolC, are at the same exact location (Figure 1).

In agreement with a low single-channel conductance in planar lipid bilayer experiments (Wong and Hancock, 2000), the native structures of OprM and TolC reveal a common closed state. It probably corresponds to the most stable conformation of this porin family in the absence of the other proteins of the pump or ligand. This closed state concerns the periplasmic entrance as well as the β-barrel exit side. Indeed, accessible diameters at the exit side, delimited by extracellular loops, are 3 Å for OprM at T105 and 6 Å for TolC at G271 (Figure 3). Yet the opening needs to be larger than 10 Å for the traffic of molecules such as antibiotics. OprM also presents a constriction formed by a triplet of leucine (L412) in van der Waals contacts, whereas the diameter of the TolC entrance is minimum (4 Å diameter) at the G365 level.

The periplasmic entrance commonly shows a constriction formed by the two α-helical coiled-coils H7/H8 called ''inner coiled-coil'' from each monomer. The other coiled-coil formed by H3/H4 is called the ''outer coiled-coil.'' According to an electrophysiological and mutagenesis analysis of TolC (Andersen et al., 2002; Bavro et al., 2008), one hydrogen bond between the inner and outer coiled-coil has been shown to be essential to maintain periplasmic entrance in closed conformation. It involves a highly conserved tyrosine localized on the inner coiled-coil, establishing hydrogen bond with an acidic residue localized on the outer coiled-coil (Y404-D205 in OprM and Y362-D153 in TolC, as shown in Figure 3). Disruption of these key interactions and opening of the porin probably involves interaction and energy from its RND and MFP partners.

Contrary to previous results (Akama et al., 2004), the present structure of OprM has a relatively low thermal factor (< 31.6 Å^2) that allowed us to localize 543 water molecules of the first hydration layer, outside as well as inside the channel, the distribution of which being different in each monomer. None of the detergent molecules has been observed in the electron density. This asymmetric water network, in particular inside the pore, could participate in substrates trafficking, but no clear pathway could be detected although some chains comprise more than five water molecules.

In contrast to the homotrimer AcrB (RND partner) where crystal structures in nonsymmetrical space group revealed different conformations between monomers (Murakami et al., 2006; Seeger et al., 2006) suggesting a peristaltic efflux system, there is very little difference between monomers of OprM or TolC. Nevertheless, we observed some local differences between monomers in the case of OprM that exclusively concern polar side chains (rmsd from 2.5 to 4 Å). These residues are R98, K187, R194, N245, R311, R341, K391, K402, R405 (the only amino acid involved in a salt bridge), and N410 (Figure 4). For four of them the differences may be attributed to either a high temperature factor (R98, R311, R341) or a poorly structured region (N245). The other five residues are localized at the periplasmic entrance, with a relatively low thermal motion. Moreover, functional studies of TolC mutants suggest that some of these residues could be key residues involved in interaction of OprM with its RND partner. According to in vitro experiment (Stegmeier et al., 2006), mutated OprM can replace TolC into a ''AcrAB-OprM'' efflux system supporting a detailed comparison between the two proteins. Superimposition of structures shows R194 from OprM to be at the same location than Q142 in TolC, a key residue

