Structure of reconstituted bacterial membrane efflux pump by cryo-electron tomography

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Complexes of OprM and MexA, two proteins of the MexA–MexB–OprM multidrug efflux pump from Pseudomonas aeruginosa, an opportunistic Gram-negative bacterium, were reconstituted into proteoliposomes by detergent removal. Stacks of protein layers with a constant height of 21 nm, separated by lipid bilayers, were obtained at stoichiometry of 1:1 (w/w). Using cryo-electron microscopy and tomography, we showed that these protein layers were composed of MexA–OprM complexes self-assembled into regular arrays. Image processing of extracted sub-tomograms depicted the architecture of the bipartite complex sandwiched between two lipid bilayers, representing an environment close to that of the native whole pump (i.e. anchored between outer and inner membranes of P. aeruginosa). The MexA–OprM complex appeared as a cylindrical structure in which we were able to identify the OprM molecule and the MexA moiety. MexA molecules have a cylindrical shape prolonging the periplasmic helices of OprM, and widening near the lipid bilayer. The flared part is likely composed of two MexA domains adjacent to the lipid bilayer, although their precise organization was not reachable mainly due to their flexibility. Moreover, the intermembrane distance of 21 nm indicated that the height of the bipartite complex is larger than that of the tripartite AcrA–AcrB–TolC built-up model in which TolC and AcrB are docked into contact. We proposed a model of MexA–OprM taking into account features of previous models based on AcrA–AcrB–TolC and our structural results providing clues to a possible mechanism of tripartite system assembly.

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1. Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium involved in severe human diseases (e.g. meningitis and septicemia). Therapeutic treatments against this bacterium found their limit because of its high resistance to a broad spectrum of antibiotics. This capability is partly due to the presence of several multidrug efflux systems, which are capable of exporting drugs out of the cell [1–3]. The MexA–MexB–OprM system belonging to the Resistance–Nodulation–Cell Division (RND) family is a tripartite complex of constitutive expression that can be induced by the presence of antibiotics [4]. MexA–MexB–OprM is composed of an inner-membrane transporter protein (MexB), an outer-membrane channel protein (OprM) and a periplasmic lipoprotein anchored into the inner membrane (MexA). MexB belongs to the 12-transmembrane helices family. It forms a trimer (330 kDa) that protrudes by 7 nm into the periplasm [5,6], MexB, a trimeric transporter is assumed to carry drugs out of the cytoplasmic or periplasmic compartments using proton motive force according to a functionally rotating mechanism [7]. OprM is a trimer (147 kDa) consisting of a 4-nm-long transmembrane domain comprising 12 strands that form a β-barrel and a 10-nm-long periplasmic domain comprising 12 α-helices [8,9]. OprM is a facilitator agent permitting the exit of antibiotics by crossing through the outer membrane. MexA (monomeric weight of 39 kDa) has been crystallized in 2004 but its structure was not completely solved due to the high flexibility of its C-terminal domain [10–12]. X-ray diffraction studies have unveiled an unexpected tridecameric structure, a hexamer facing a heptamer facing a head-to-head manner [10,11]. More recently, the structure of MexA has been almost completely determined, depicting the membrane-proximal domain as a β-roll [13]. From this newly depicted structure of MexA, a model of the AcrA–AcrB–TolC efflux pump with a stoichiometry AcrA3–AcrB3–TolC3 has been proposed. Accordingly, our previous works on MexA–OprM interaction monitored by Quartz-Crystal Microbalance with Dissipation monitoring suggested an OprM2–MexA3 stoichiometry [14].

Even though models of the tripartite pumps were proposed [13,15], there are no direct observations of the pump or a part of it anchored
between two lipid bilayers. Due to this specific location of the pump, before considering the reconstitution of the whole pump, we focused on the reconstitution of a bipartite MexA–OprM complex as a first step. Indeed, strong interactions between OprM and MexA have been reported unlike OprM and MexB [14,16]. Using the reconstitution method based on detergent removal, we describe here the formation of MexA–OprM complexes inserted between two lipid membranes. As these complexes were arranged in a multilayered stack, we determined their architecture by both cryo-electron microscopy and cryo-electron tomography representing the suitable technique for structural analysis of micrometer-sized assemblies at a nanometer scale [17,18]. The present study provides new insights about the interaction between OprM and MexA in the context of the whole pump assembly.

2. Materials and methods

2.1. Materials and reagents

1,2-Dioleoyl-sn-glycero-phosphocholine (DOPC) and octyl-β-D-glucopyranoside (β-OG) were respectively purchased from Avanti Polar Lipids (USA) and from Sigma.

2.2. Preparation of OprM and MexA proteins

Expression and purification of the transmembrane protein OprM were achieved as previously described [19]. The MexA lipoprotein expression and purification were performed following a similar protocol. Briefly, the membrane envelopes from broken Escherichia coli cells were solubilized in 20 mM Tris–HCl pH 8.0, 10% glycerol (v/v), 15 mM imidazole and 75 mM β-OG, overnight at 20 °C. The (poly)His-tagged solubilized membrane proteins were loaded onto a Ni-NTA resin column and were then eluted with a linear gradient of imidazole (60–500 mM). OprM (or MexA)-containing fractions were pooled and concentrated to 5 mg/ml. Finally, the purified membrane protein OprM (as well as MexA) was exchanged for suitable buffer by dialysis, in the presence of 20 mM Tris-β-glycero-phosphocholine (DOPC) and octyl-β-OG.

2.3. Preparation of unilamellar vesicles

Lipids were dissolved in chloroform, dried under vacuum with a desiccator for 1 h. Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation, followed by sequential extrusion through 0.4, 0.2 and 0.1 nm Nucleopore filters [20]. Small unilamellar vesicles (SUV) were obtained by an alternated 45 min sonication of the LUV with a tip sonicator (Branson, Digital Sonifier 250) as described previously [21].

2.4. Reconstitution of OprM and MexA proteins into proteoliposomes

DOPC vesicles (25 μg/ml) were added in a buffer consisting of 10 mM Hepes pH 7.4 and 150 mM NaCl (final volume 500 μl). Then, β-OG was introduced to a final concentration of 20 and 30 mM for MexA and OprM experiments, respectively. For experiments involving both proteins, a 30 mM β-OG concentration was used. Before proteins were introduced, the solution was gently stirred for 15 min at 4 °C for homogenization. Finally, MexA and/or OprM proteins were introduced at desired OprM to MexA ratios (w/w) (final protein concentration was 10 μg/ml). After an incubation time of 1 h under gentle agitation at 4 °C, 50 mg/ml of polystyrene beads (SM2 Biobeads, Biorad) were added to remove the detergent. Biobeads were picked out of the solution after an overnight incubation time at 4 °C under gentle stirring.

2.5. Cryo-electron microscopy (cryo-EM)

A 5-μl sample was deposited onto a holey carbon-coated copper grid (Ted Pella, CA,USA). The excess of the solution was manually blotted with a filter paper (Whatman Filter paper). Samples were frozen into liquid ethane using Leica EMCP equipment and the grids were mounted onto a Gatan 626 cryoholder, transferred into the microscope, and kept at a temperature of about −175 °C. Samples observations were performed with a Tecnai-F20 FEG microscope operating at 200 kV (FEI, The Netherlands). Low-dose images were recorded at a nominal magnification of 50000 with a 2k×2k USC1000 slow-scan CCD camera (Gatan, CA, USA).

2.6. Cryo-electron tomography (cryo-ET)

Cryo-electron tomography (cryo-ET) experiments of MexA–OprM proteoliposomes (R = 1:1) were collected on a Tecnai G2 Polara microscope (FEI, The Netherlands) operating at 300 kV and equipped with a Gatan postcolumn GIF 2002 energy filter, using UCSF tomography software. The nominal magnification used was 27,500 and images were recorded on a two times binned 4k×4k CCD camera from −69° to +69° with a 1.5° step scheme at a defocus level of about −4.0 μm. Final tomograms consisted of 93 images with an electron dose per tomogram evaluated at 60 e⁻/Å².

2.7. Tomograms reconstruction, image analysis, and modeling

Alignments and weighted back-projection-based reconstructions of raw tilt series using −20 fiducials were computed with EtoMO (IMOD package, Laboratory for 3-D Electron Microscopy of Cells, Boulder, Colorado, USA) [22]. Windowing of sub-tomograms containing seven MexA–OprM complexes (one central and its 6 neighbors) was carried out from three different missing wedge oriented tomograms. Then, 3D alignment (rotation and translation) performed on the central complex was computed with SPIDER (Wadsworth Center, New York, USA) [23]. The sub-tomogram average only displayed the central complex after applying a cylindrical mask.

Molecular graphics images were produced using either the UCSF Chimera package (Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco) [24] or AMIRA (Mercury Computer Systems).

Segmentation of Fig. 2A was carried out by applying several cycles of median 3 × 3 × 3 filters using IVE/Prism package [25] and then a threshold in such a way that the lipid membranes appear contiguous. Then, with ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2009), several cycles of opening were applied in order to only keep lipid bilayer features. Finally, this mask was used to segment the objects of interest inside and outside of the flattened liposomes.

Modeling of MexA–OprM complex is based on the AcrA3–AcrB1–ToLC model of Symmons et al. [13] used as a template. First, the OprM structure (PDB: 3D5K) was 3D aligned onto ToLC using SSM [26] (at EBI server). Second, using the coordinates of MexA (PDB: 2V4D), we modeled an “extended” MexA similar to that of AcrA from AcrA3–AcrB1–ToLC model. For that we proceeded by aligning separately the four domains of MexA on those of AcrA (using Coot built-in SSM command) and then linked back one to each other using stereochemical regularization tools available in Coot [27]. Finally, we adjusted the interface between this “extended” MexA and OprM in order to put amino acids involved in the MexA–MexB–OprM formation [28] face to face. The final OprM₁₂–MexA₁ model was calculated by imposing a 3-fold symmetry, and submitted to simulated annealing using the standard protocols implemented with the software Phenix [29].

3. Results

3.1. MexA–OprM complexes incorporated into two opposing lipid membranes

OprM and MexA proteins were both purified and solubilized in the presence of β-OG as previously described [14,30,31]. OprM is a
transmembrane protein requiring 30 mM βOG whereas MexA is anchored into a lipid membrane with a palmitoyl acyl chain at the N-terminal amino acid sequence and maintains solubility in the presence of 20 mM βOG. Based on our previous QCM-D (Quartz-Crystal Microbalance with Dissipation monitoring) study, which provided evidence for strong interactions between OprM and MexA proteins [14], both proteins were mixed in a 1:1 (w/w) ratio in the presence of 30 mM βOG to promote complex formation. Incorporation of these proteins into lipid membranes relied on the detergent removal from a lipid-protein-detergent micellar solution. Cryo-EM observations of reconstituted materials (Fig. 1A and B) revealed stacks of protein layers (white arrowheads) with a regular thickness of 21 nm, alternating with lipid bilayers (black arrowheads). These regular stacks made of MexA–OprM complexes incorporated into lipid bilayers were formed at various pH values (from 6.5 to 8.2) and in the presence of 150–450 mM NaCl without noticeable change of the overall organization of stacked protein layers.

Interestingly, varying the OprM to MexA ratio modified the regular organization of protein complexes. Indeed, at an OprM to MexA ratio of 1:2 (w/w), stacks of protein layers exhibiting a 26-nm-thick protein layers were formed and co-existed with the 21 nm protein layers previously observed at the 1:1 ratio (Fig. 1C). Clearly, MexA molecules added at a 1:2 ratio were involved in the formation of MexA–OprM complexes forming 21-nm-thick patches and those not interacting with OprM formed a MexA–MexA protein assembly of 26 nm. To support this hypothesis, MexA was reconstituted solely into lipid bilayers. Cryo-EM revealed the formation of 26-nm-thick protein layers sandwiched between two lipid membranes (Fig. 1D). Straight features visible on side-view images (Fig. 1D, white arrows) and annular structures on top-view images (Fig. 1E, white circles)

Fig. 1. Cryo-EM images of membrane reconstitution of OprM and MexA at various ratios. A–C) Proteoliposomes made of stacked protein layers at a OprM:MexA ratio of 1:1 (A and B) and 1:2 (C). A and B) 21-nm-thick protein layers (white arrowheads) separated by lipid bilayers (black arrowheads). C) The addition of MexA results in the creation of 26-nm-thick protein layers in addition to the 21-nm-thick ones. D and E) Respectively, side and top views of the membrane reconstitution of pure MexA protein. MexA proteins are present at the periphery as 13-nm-high structures (black arrows) and form in stacks MexA–MexA assemblies of 26 nm (black rectangle). F) Comparison of OprM–OprM, MexA–OprM and MexA–MexA membrane reconstitutions leading to 13, 21 and 26 nm protein layers, respectively. Scale bars: 25 nm.
suggested that MexA proteins formed a cylindrical-shaped assembly. In addition, apart from these 26-nm-thick assemblies, 13 nm protein densities protruded out of the membrane at the edge of the proteoliposomes (Fig. 1D and E, black arrows) and resembled those observed on supported lipid membranes in our previous work [31]. Their superimposition in a face-to-face orientation likely triggered the formation of 26-nm-thick MexA–MexA assemblies (Fig. 1E, black rectangle) sandwiched between two membranes quite similar to what was described with a soluble MexA mutant [10,11].

Likewise, for a ratio of 1:2, a MexA–MexA assembly was measured with a thickness of 26.5 nm ± 0.2 nm from cryo-EM images (Fig. 1F, right). In addition, at a ratio of 2:1, 13.4 nm ± 0.1 nm protein stacks were formed along with the 21.2 nm ± 0.2 nm MexA–OprM stacks (Fig. 1F, left). This new assembly corresponded to OprM–OprM stacks incorporated into the lipid bilayer with an up-and-down orientation according to our previous structural study of OprM [30]. Thus, the thickness of the protein layer of 21.2 nm ± 0.2 nm was specifically ascribed to the MexA–OprM assembly (Fig. 1F, middle) and provided evidence that by mixing OprM and MexA in solution, an MexA–OprM complex was reconstituted between two opposing lipid membranes as recently measured in detergent L3 phase [32]. Moreover, the fact that in our experimental conditions, the constant thickness of MexA–OprM layers was obtained at a 1:1 ratio suggested that a single type of assembly was formed with a set number of OprM and MexA proteins per complex. It also suggested that the formation of the complex involved an equimolar ratio of MexA and of OprM owing to their relatively close molecular weights (respectively 39 and 49 kDa per monomer).

3.2. Arrangement of MexA–OprM complexes within proteoliposomes studied by cryo-ET

For a better understanding of the MexA–OprM complex arrangement, we used cryo-electron tomography (cryo-ET) to study the 3D architecture of the proteins sandwiched between two lipid membranes. Among 31 tomograms, proteoliposomes were mainly observed along two perpendicular orientations within the ice layer, providing top and side views of the MexA–OprM complex. The different orientations of the proteoliposomes allowed us to fully explore the arrangement of the MexA–OprM complex with respect to the lipid membranes despite the missing wedge effect (Fig. 2).

Likewise the typical cryo-EM images shown in Fig. 1, a 3D representation of a segmented side-view tomogram (i.e. orientation

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**Fig. 2.** Arrangement of MexA–OprM complexes into proteoliposomes analyzed by cryo-ET. A) 3D representation of a side-view tomogram. Lipid bilayers are in red. Protein densities in blue and light blue. B) A 0.42-nm-thick tomographic slice extracted from the same tomogram. Rod-like structures correspond to MexA–OprM complexes. Note the faint dark line (black arrows) corresponding to MexA anchoring into lipid bilayer. C) Schematic drawing of MexA–OprM complexes arrangement into proteoliposomes. D) A 0.42-nm-thick tomographic slices extracted from a top-view tomogram displaying a protein density array. E) 2D averages of sub-images from 2.4 nm extracted sections along Z' axis above (1), within (2) and below (3) the lipid bilayer revealing the up and down arrangement of OprM molecules. OprM in (1) is located in the centre of three OprM in (3) as shown after superimposing their gravity centres colored in red and green, respectively (4). The unit cell dimensions are 8 nm x 8 nm γ=60°. Scale bars: 25 nm in B and D.
perpendicular to the plane of the lipid bilayer) depicted the overall architecture of four flattened proteoliposomes exhibiting MexA–OprM complexes in and outside the proteoliposomes (Fig. 2A). A 0.42-nm-thick central slice extracted from this tomogram clearly showed 20-nm-long rods, most often regularly spaced, corresponding to MexA–OprM complexes sandwiched between two lipid membranes. Interestingly, the proteoliposomes displayed a less dense line close to their lower lipid membranes (Fig. 2B, black arrows) that was typically observed on 2D images of MexA membrane reconstitutions (Fig. 1). Note that unlike OprM which is an integral membrane protein, MexA is a surface protein merely anchored to the lipid membrane via its lipid tail. The demarcation between protein and lipid densities was more pronounced by the defocus used for the data collection (close to −4 μm) inducing this less dense line. The presence of the latter suggested that large patches of MexA–OprM complexes with a parallel orientation were formed within the stacked proteoliposomes, as schematically drawn in Fig. 2C. In support to these observations, regular protein arrays were observed on top-view tomograms as shown on a 0.42-nm-thick tomographic slice extracted after rotation angle of 32° from the 0° tilt (Fig. 2D). To understand the protein arrangement with respect to the lipid membrane, a 2.1 nm slice was extracted along the Z direction (perpendicular to the lipid membrane of the proteoliposome shown in Fig. 2D) and 131 sub-images were submitted to a X-μm slice extraction (Fig. 2E). The protein array is composed of a hexagonal packing of circular-shaped densities with a measured spacing of 8 nm in agreement with our previous 2D electron crystallography analyses of OprM and OprN proteins [30]. Moreover, to analyze the organization of OprM moiety with respect to the lipid membrane, eleven 2D averages of the next slices along Z axis were computed using the results of the previous 2D alignment (Fig. 2E1–3). OprM proteins had an up-and-down orientation with respect to the lipid membrane and were arranged in such a way that the upper OprM protein was located into the centre of the three OprM proteins (Fig. 2E4). On the basis of these results, the MexA–OprM complexes are oriented in opposite directions on both sides of the membrane (Fig. 2C).

To conclude, this tomographic exploration indicated that MexA–OprM complexes which possess a cylindrical-shaped structure are assembled with a parallel orientation and form patches of several tens of molecules. It is likely that the MexA–OprM complexes are formed during detergent removal, which is consistent to our QCM-D experiments providing evidence of interactions between OprM and MexA in the presence of detergent [14]. One possible mechanism leading to the stack of proteoliposomes could arise from OprM assembly. Since a stacked-layer organization was observed for both proteins, it is difficult to determine which protein is responsible for this assembly. However, the alternate orientation of transmembrane OprM could coerce the formation of patches of parallel-oriented complexes (Fig. 2C).

### 3.3. Towards molecular details of MexA–OprM complexes

To gain more structural details on MexA–OprM complexes, we carried out sub-tomogram averaging. A selection of 919 sub-tomograms (526 top and 393 side views) extracted from three tomograms was submitted to an iterative alignment procedure. Since the defocus difference has been estimated to be less than 1 μm, it seemed acceptable to merge data from both top and side views in order to reduce the missing wedge limitation.

The average of sub-tomograms depicted the protein complex sandwiched between two lipid bilayers (LB) and clearly displayed an asymmetric membrane anchoring (Fig. 3). At the upper LB, the continuity of densities between LB and the complex indicated a full insertion via its integral membrane protein OprM, whereas the apparent discontinuity at the lower LB reflected a rather mere interaction of MexA protein moiety with the LB (Fig. 3A and B). The protein complex formed a 13 nm hollow cylinder on almost all of its length (Fig. 3C and D), its lower part being less clearly defined. To more accurately determine the location of OprM inside the complex structure, a 3D density map of the OprM atomic model has been fitted into our sub-tomogram average. The trimeric OprM density map has been generated from the atomic model (PDB ID: 3D5K) and filtered to 3 nm resolution using Xmipp procedure. Then, the precise location of OprM was determined using a 3D cross-correlation function allowing us to fit OprM map into cryo-EM complex average (Fig. 3E).

Interestingly, bulges located at 2.4 nm below the LB (Fig. 3A) perfectly matched the region containing a ring of two α-helices (H1 and H5) and loops forming an equatorial domain close to OprM β-barrel domain of

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Fig. 3. Sub-tomogram averaging of MexA–OprM complexes. A) Coronal slice through the masked average of the sub-tomograms. B) Isosurface visualization of the MexA–OprM complex sandwiched between two lipid bilayers (LB). Note that the use of a cylindrical mask for displaying the central complex cut arbitrarily the lipid bilayers and the lower densities of MexA, likely resulting from both central and neighboring MexA. C and D) Axial slices through the average of the sub-tomogram showing the central complex with cylindrical shape surrounded by six neighboring complexes. Their positions (dashed lines) with respect to the isosurface shown in B are indicated. E) Semi-transparent isosurface with the OprM molecule fitted onto the density. Distances in nm. Scale bar for A, C and D: 5 nm.
the atomic model [Fig. 3E, asterisk] [8]. This OprM fitting provided the accurate boundaries of the OprM trimer, indicating that extra densities protruding out of the OprM map correspond to MexA proteins. Indeed, the MexA densities produced a 4.2 nm length cylindrical structure below OprM (Fig. 3E). This part is however composed of weaker densities than that of OprM as shown in Fig. 3A and D. Beyond this cylindrical part, MexA densities widened in an area of 8-nm-thick composed themselves of two densities strata, but could not be identified as isolated domains. That could be related to the fact that the domains are highly flexible as suggested by Vaccaro et al. [12] and Symmons et al. [12,13]. Despite several attempts at using sub-tomogram classifications (principal component analysis, self-organizing map technique and variants) or different alignment strategies, we did not succeed in identifying homogeneous sub-populations clearly revealing MexA domains. We may notice that it was all the more difficult since the neighboring MexA were very close to each other and thus, the boundaries of these MexA domains cannot be easily traced.

4. Discussion

In the present work we present the membrane reconstitution of OprM and MexA. We found conditions that promote the formation of MexA–OprM complexes amenable to structural studies using cryo-ET. We showed that MexA–OprM complexes are anchored on both extremities to a lipid membrane providing an almost native environment. MexA–OprM complex has an overall measured height of 26 nm as presented in Fig. 3E. Since the periplasmic moiety of OprM protrudes out from the membrane by about 10 nm, this raises questions about the height of MexA and its flexibility. Our current cryo-EM observations of MexA incorporation into a supported lipid bilayer [31] revealed a 13 nm layer of MexA densities which was about 4 nm greater than the height of the structure published in 2004 [10,11]. It was pointed that this extra height could correspond to the unsolved domain in contact with the lipid bilayer, corresponding to one third of the total protein. Recently, the N- and C-termini of MexA have been solved as a compact domain, termed the membrane-proximal domain, of 4.5 nm height and close to the inner membrane [13], confirming our previous observations.

Unlike OprM molecules whose trimeric structure solved by X-ray is considered as the functional form, MexA structure has been solved in a hexameric/heptameric assembly for which there is no functional evidence. Recently an assembled structure of the complete tripartite pump AcrA–AcrB–TolC has been presented based on cross-linking approach [13]. The interaction of the AcrA periplasmic adaptor with the AcrB transporter required large flexions from the X-ray model of MexA, structurally close to AcrA, indicating that the monomeric form of MexA/AcrA could adopt various structural conformations that are dependent on the partner with whom it interacts.

In this context, we seek to understand the arrangement of MexA molecules within our tomographic map of MexA–OprM complex. The model of the complete tripartite pump AcrA–AcrB–TolC proposed by Symmons et al. [13] is attractive, even though its intermembrane distance of ca. 17 nm (10 nm for the TolC extracellular domain + 7 nm for AcrB) is shorter than our 21 nm measured distance. Indeed, it proposed an extended structure of AcrA more compatible with our cylinder-shaped structure than the crystal structure (PDB 2V4D). We manually fitted the bipartite TolC–AcrA model built from the tripartite model (Fig. 4A). It appeared that while the TolC moiety and α-hairpin domain of AcrA fit well, the last three C-terminal domains of AcrA which form a flared structure, failed to fit into the tomographic map. The absence of the third component MexB may explain in part this

Fig. 4. Model of the MexA–OprM complex. A) Fitting of AcrA–AcrB–TolC model of Symmons et al. [13] into EM map. B) Proposed model of MexA–OprM complex based on AcrA–AcrB–TolC model and biochemical studies of MexA–OprM interaction [28]. The membrane-proximal and β-barrel domains of MexA are localized within densities adjacent to the lipid bilayer. C) Model of MexA–OprM complex colored in red and yellow, respectively. D) Enlarged view showing details of MexA–OprM interface (marked with a black rectangle in C). The α-helical hairpin of MexA was docked in OprM coiled coils in a similar way to Symmons model. Amino acids (represented by spheres and numbered according to protein sequence) involved in the MexA–OprM interaction according to [28] were used to build model. E) Scheme of a hypothetical mechanism of the assembly of the tripartite pump. The insertion of the MexB/AcrB third component into a MexA–OprM complex could trigger a locking mechanism bringing OprM and MexB in contact. This model was oriented in such a way that the upper and lower lipid bilayers correspond to the outer (OM) and inner (IM) membranes of bacteria, respectively.
mismatch. As expected, the membrane-proximal domain of AcrA was partly located in the 8-nm-thick layer that is affixed to the lipid membrane.

To increase the fit, we built a bipartite MexA–OprM model based on the following constraints: (i) an extended structure of MexA (derived from the AcrA of Symmons et al. [13]), (ii) the OprM model (PDB 3D5K), (iii) a trimmer arrangement OprM3–MexA3, as proposed by previous models [13,15] and in accordance with our present complex reconstitution at a ratio of 1:1 (w:w) and mass measurements using QCM-D [14,31], and (iv) biochemical evidence that A108 and V129 (for MexA sp: P52477) and G216, G242, T198 and F439 (for OprM sp: Q51487) were involved in the MexA–OprM interaction [28]. The model was assembled in such a way that the residues T198, F439 and G216, G424 of OprM were close to V129 and A108 of MexA, respectively (Fig. 4B–D). The overall length of this MexA–OprM model is suitable to the intermembrane distance of our tomographic map (Fig. 4B). Although the lipoyl domain remains outside of the tomographic map, the β barrel and membrane-proximal domains of MexA are now located in the two strata of the 8-nm-thick layer. In addition, the MexA structure is incomplete since 12 residues from the N-terminus and 20 residues from the C-terminus remain unsolved. These ‘missing’ regions could fill in the extra distance between MexA and the lower lipid bilayer that is also of low density in our tomographic map.

It is worth noting that for an intermembrane distance of 21 nm, we build a model with a surface of interaction OprM/MexA smaller than that of ToIC/AcrA which leads us to make two assumptions. First, the difference in height would be specific of each species and thus their tripartite complex would have an intermembrane distance of 17 nm for E. coli and 21 nm for P. aeruginosa. Second, the absence of MexA and OprM transporter could explain the difference in height. The assembly mechanism as proposed by Misra and Bavro [33], supposed an intermediate step comprising the formation of an MexA–OprM bipartite complex. According to our data this complex would have a 21 nm intermembrane distance. Then, the binding of MexB interacting with MexA adaptor to build the whole pump could trigger a slight shift in helical–helical interactions of OprM–MexA, leading to a tripartite complex of a 17-nm high between the two lipid membranes. In addition, a MexA conformational change re-orienting on solid support and reconstituted into a lipid membrane, Langmuir 23 (2007) 507–517.

5. Conclusion

We were able (i) to reconstitute proteoliposomes containing MexA–OprM complexes, with both proteins anchored in two opposing lipid membranes similarly to their organization in the periplasmic space of P. aeruginosa, visualized here in cryo-EM and (ii) to discriminate between different types of proteoliposomes (i.e. OprM, MexA and OprM–MexA). The tomographic map of MexA–OprM complex allows us to undoubtedly identify OprM and to locate MexA molecules. Based on the intermembrane distance, we proposed a model of MexA–OprM complex providing new insight on the assembly of whole efflux pumps. Our reconstitution method and the structural characterization approach described in the present work allow us to consider further studies of reconstitutions including MexB, the third component that would provide the structure of the whole MexA–MexB–OprM system.

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