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Homology model of the multidrug transporter LmrA from *Lactococcus lactis*

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Abstract—LmrA is an ATP dependent multidrug transporter from *Lactococcus lactis* conferring antibiotic resistance to 17 out of 21 most frequently administered antibiotics. Starting from the dimeric crystal structure of *Vc*-MsbA, we built two homology models, with NBD:NBD interfaces reflecting the nonenergized and energized state, respectively. The TMD:TMD topology of the dimer is consistent with the previously obtained substrate photoaffinity labeling pattern suggesting binding of substrates at the TMD:TMD interface involving helix 3 of one monomer and helices 5 and 6 of the other monomer.

1. Introduction

The phenomenon of multidrug resistance represents one of the most significant problems in the treatment of cancer with cytostatics and of infections with antibiotics. Overexpression of membrane bound ATP dependent drug efflux pumps such as the multidrug efflux transporter LmrA from *Lactococcus lactis* results in an accelerated efflux of multiple classes of drugs including a broad range of clinically used antibiotics.[1] Inhibitors of this pump have been shown to reestablish drug sensitivity.[2] LmrA is a structural and functional homologue of the human multidrug transporter P-glycoprotein (Pgp). It consists of 590 amino acid (~64 kDa) membrane pump and was the first ABC transporter to be identified in a procaryotic organism.[3] It is considered to be a half transporter, which is functionally active as homodimer.[4] The basic blueprint of ABC transporters consists of four core domains. Two transmembrane domains form a tunnel through, which solutes can move. These transmembrane domains usually consist of six membrane spanning α-helices that contain the substrate binding sites. In addition, ABC transporters possess two highly conserved nucleotide binding domains (NBDs) containing the ATP-binding and -hydrolyzing 'motor domain' of the transporter. Multidrug ABC transporters are responsible for the import and export of a vast array of substrates. Neither the 3D-structure of the binding domain nor the mechanism of transport are well understood at present. Protein structural data are an important prerequisite to understand the molecular basis of transport and thus of target based inhibitor design.

In absence of high resolution structural data for LmrA one alternative is to generate a homology model based on the structure of a known sequence homologue. To date only the crystal structures of three bacterial transporters have been crystallized with their TMDs, the Lipid A transporter MsbA from *Escherichia coli* (*Eco*-MsbA, pdb code: 1JSQ)[5] and from *Vibrio cholerae* (*Vc*-MsbA, pdb code: 1PF4)[6] and the Vitamin B12 transporter BtuCD from *E. coli* (pdb code: 1L7V).[7] In addition several isolated NBDs have been crystallographically resolved including HisP,[8] MalK,[9] TAP1,[10] MJ0796,[11] MJ1267,[12] Rad50,[13] and HlyB.[14] Very recently the crystal structure of the isolated LmrA NBD became available (pdb code: 1MV5). Unfortunately the work with details on crystallizing conditions and interpretations of the structure still remains to be published. Evaluation of the three full length transporters

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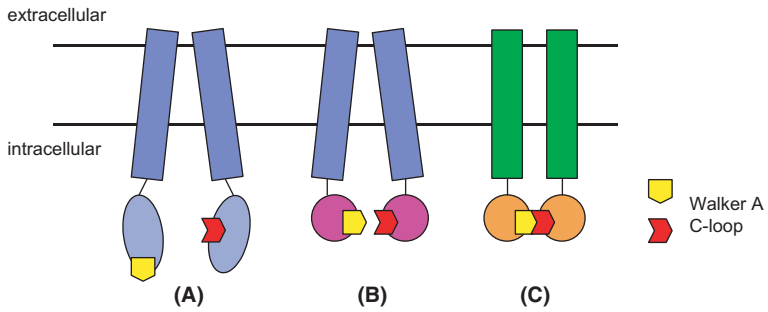

Figure 1. Stepwise generation of the atomic detail models for LmrA; motifs involved in nucleotide binding are depicted in yellow (Walker A) and red (C-loop); A—model 1; B—model 2 with NBD modeled on TAP1, reflecting nonenergized state; C—model 3 NBD in its ATP bound state.

identified *Vc*-MsbA as the most suitable template for modeling the transmembrane regions, since BtuCD has 20 transmembrane segments while LmrA has a predicted number of six TMs. Though this precludes BtuCD from being used as a full length template, the NBDs of BtuCD exhibit a polypeptide fold similar to that observed in isolated NBDs.[15]

Vc-MsbA not only shows overlapping substrate specificity with LmrA, but also yielded highest scores in BLAST sequence alignment results (30% identities, 48% positives). The TMD:TMD interface in this template also represents a more likely conformation than the crystallographic interface observed for *Eco*-MsbA. The only drawback of the crystal structure is that the N terminal part of the NBD is folded in a way that is not consistent with previously resolved NBDs. This might be due to the crystallographic conditions used and might not necessarily represent a physiologically relevant conformation of the motor domain. The N-terminal part of the β-domain of the NBDs is detached from the adjacent α-domain. In contrast to crystal structures of isolated nucleotide binding domains[8–12] and evidence from cross-linking studies of P-gp[16] the Walker A loop of one motor domain and the C-loop of the other motor domain do not face each other (Fig. 1A).

2. Results and discussion

In a first step we built a homology model of LmrA using the novel template structure of *Vc*-MsbA (Fig. 1, model A). Alignments obtained by the 'Align123' module of the software package InsightII[17] were checked for deletions and insertions in structurally conserved regions and, if necessary, corrected manually.[18] The resulting LmrA model correctly predicted polar amino acid residues in TM segments to be oriented towards the central pore, while apolar residues face the lipid bilayer, supporting a valid sequence alignment. As mentioned above the NBD:NBD interface as shown in this model, fails to explain the catalytic requirements expected for an ABC transporter. The NBDs, though properly folded, are in a position, which does not satisfy data obtained by kinetic studies,[19,20] FRET,[21] cross-links of the homologous transporter P-gp[16] and crystal structures of isolated NBD interfaces.[15] Hence we replaced this NBD by an NBD generated by using the monomeric TAP1 as template (Fig. 1, model B; Fig. 2). Of all available NBDs, TAP1 exhibits the highest sequence identity with the NBD of LmrA (35% identity; 55% strong similarity). Sequences of the LmrA NBD and Tap1 were aligned in the Align123 module of InsightII, using the BLOSUM 62 matrix with a gap penalty of 11 and a gap extension penalty of 1. The alignment was manually optimized and the model constructed. Subsequent splice repair was performed with a cycle of 500 steps Steepest Descent Algorithm minimization. The NBD model was fitted by least square superpositioning of its Walker A motif coordinates onto the corresponding motif in the BtuCD crystal structure resulting in a comparable NBD:NBD interface (Fig. 1, model B; Fig. 2). In order to correctly reestablish the TMD:NBD interface the ICL1 (intracellular loop 1) connecting TM helices 2 and 3 of each monomer was manually brought into position. The final model assembly was created by recalculating the linker region between TM helix 6 and NBD and subsequent splice repair with a cycle of 100 steps Steepest Descent Algorithm minimization. The 3D model of the nucleotide binding domain was checked in terms of geometry using ProStat and Verify 3D[22] pull down of InsightII.

This model now shows an NBD:NBD interface satisfying experimental data whereby the motifs involved in ATP binding and hydrolysis are oriented towards the interface. Since the BtuCD crystal structure has been obtained in the absence of ATP, the model structure might be assumed to reflect the nonenergized state.

In order to allow the generation of a model reflecting the energized state a third model was obtained by superposition of the NBDs of model B onto dimeric MJ0796 (pdb code: 1L2T), which has been crystallized in the presence of ATP. The closed conformation of the NBDs required reorientation of the TMDs yielding a closed TMD:TMD interface and a consensus NBD:NBD interface (model C). TMD repositioning was achieved by changing the tilt angle of the TMDs towards each other without altering the accessibility of helices to the central aqueous pore. The reliability of the obtained protein folding was assessed by using ProStat and Ramachandran plots. 98.5% were in allowed regions of the Ramachandran plot.

In the process of preparation of this manuscript the coordinates of the crystal structure of the LmrA NBD

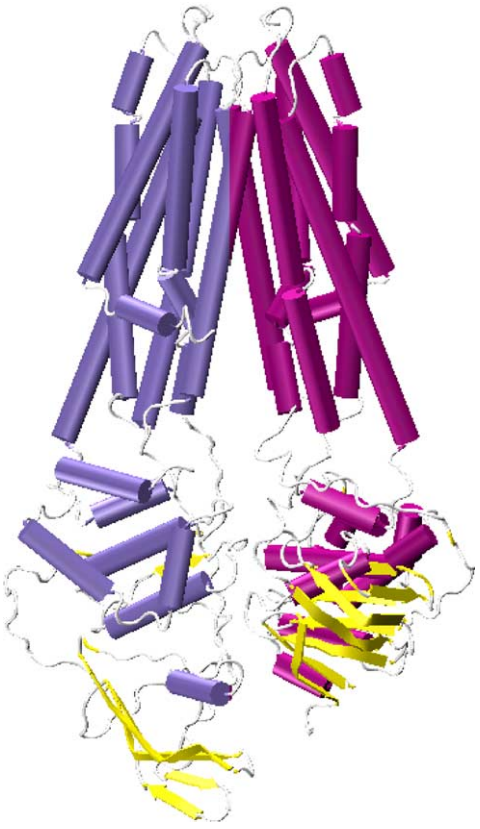

Figure 2. Comparison of model 1 (left) and 2 (right).

became available. The unit cell shows four NBD monomers and two molecules of each ADP and ATP. The Y-loops of two adjacent NBD monomers are oriented towards each other sandwiching both an ATP and an ADP molecule between each other.[23] The pseudotetrameric structure is thus highly unlikely to represent a physiological state of the LmrA NBD. As details on the crystallization conditions await publication, we decided to restrict our considerations to the level of the individual monomer. The RMS fit of the four monomers yielded deviations below 2.3 Å, whereby two monomers showed close similarity with an RMSD value of below 1 Å. A comparison of each of these monomers with the NBD portion of models B and C gave an overall RMSD of 3.3 Å. Considering a rigid body movement of the α-helical domain relative to the F1-type core domain[14,24] during the catalytic cycle led us to perform a superposition of these NBD subdomains individually. When excluding the D-loop (which deviated within the four monomers of the crystal structure) and the adjacent carboxy terminal α-helix (which deviated between the crystal structure and the model) the core domain showed an RMSD of 1.1 Å. The α-helical domain deviated by 2.2 Å. This indicated valid template selection.

There is general agreement that in multidrug transporters substrate binding and transport is mediated by the transmembrane domains. In our models, the TMDs are predicted to form a helical bundle, which lines a central aqueous pore with access to the extracellular space. The central pore is lined by TM helices 1, 3, 5, and 6 of each monomer. TM helices 2 and 4 are shielded from the aqueous environment by the other helices and are largely membrane embedded. The TMD:TMD interface is formed by helices 3 and 5 of different monomers (Fig. 3). The TMD's orientation towards each other is consistent with cross-linking data for P-glycoprotein.[25]

Previous substrate photoaffinity labeling experiments by our group indicated helices 3, 5, and 6 to be involved in substrate binding of propafenone type ligands.[26] These helices thus form two putative substrate binding domains located at the interface between the monomers. Each binding domain is formed by TMD 3 of one monomer and TMDs 5 and 6 of the other monomer. The homology model supports this hypothesis showing the close spatial proximity of these three helices. Additional support for an involvement of helices 5 and 6 in substrate binding comes from photolabeling experiments with Rhodamine 123.[27]

LmrA transports 17 out of the 21 clinically most frequently used antibiotics. Therefore it represents a paradigm for other microbial drug efflux transporters, such as cdr1, pdr5, and snq2. Microbial drug resistance represents a major health problem. Resistant strains are

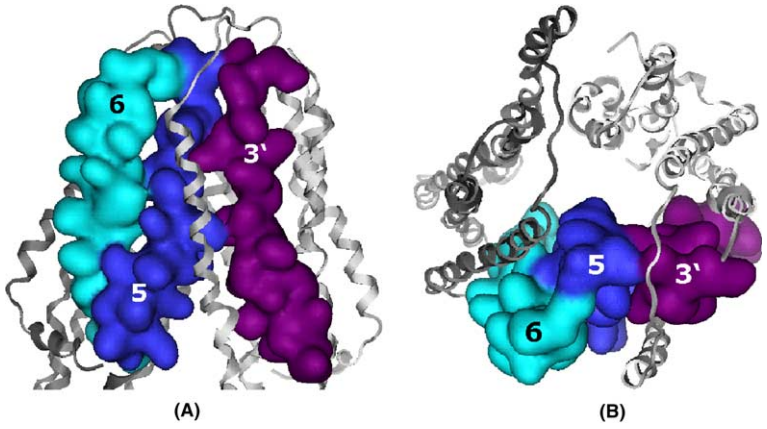

Figure 3. TMD of model 2; helices 5 and 6 of one and 3′ of the other monomer are depicted with molecular surface representation. A—side view; B—top view.

rapidly emerging and only a small number of structurally and functionally new antibiotic drugs are currently under development.[28] One promising approach in the battle against multiresistant infectious disease caused by microbes overexpressing drug efflux pumps, is the inhibition of these pumps. In the therapy of multiresistant tumours this strategy has developed to the point of clinical phase 3 studies.[29] In contrast, in the microbial field development of efflux pump inhibitors (EPIs) is still in the preclinical phase.

Despite extensive efforts, the molecular basis of drug binding and transport by multidrug transporters remains elusive. We have generated the first 3D-atomic detail model for a bacterial multidrug transporter. This model not only shows a likely NBD:NBD interface but also positions the TMDs in a manner, which is consistent with existing photolabeling and cross-linking data. The homology models thus provide us with the unique possibility to identify residues to be targeted in site directed mutagenesis. Combination with cross-linking and photoaffinity labeling experiments will provide a tool in the hypothesis driven search for substrate-interacting amino acid residues, which will guide the target based development of new EPIs.

Acknowledgements

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