Structural Characterization of the Drug Translocation Path of MRP1/ABCC1


Abstract: The rapid clearance of drugs from human cells is carried out by MRP1 and other proteins of the ABC transporter superfamily. Selective mutations carried out by De-Gorter indicated that replacement of Y324 by phenylalanine (but not by tryptophan or alanine) enhances the capacity of the protein to extrude various drugs. In this study we investigate the effect of mutation on the structure of the isolated transmembrane domain of MRP1 through molecular dynamics simulations of the protein embedded in a POPC membrane. The simulations reveal a persistent tendency of the translocation path to experience a partial constriction, losing ~50% of the water inside the conducting path. While the Wt, Y324W and Y324A transporters all experienced the same constriction, the Y324F transporter, the one having a higher clearance rate than the Wt, retains a fully open configuration. The structure of the Y324F mutant reveals an alternate set of stabilizing interactions that force a kink in transmembrane helix 6, which keeps the protein in a fully open outward-facing configuration thus providing a molecular-level account for the higher activity of the mutant. The ability of the simulations to corroborate the experimental observations implies that the homology model of MRP1 is a proper representation of the internal interactions between the residues in the protein, and can be used as a reliable model for studying the human multidrug resistance function of the MRP1 protein.

Keywords: antitumor agents · membrane proteins · molecular dynamics · multidrug resistance · mutagenesis

1 Introduction

Cancerous tumors have long been known to develop resistance to a variety of drugs, allowing them to evade cellular death. Traditional chemotherapy treatment consists of various types of anticancer agents, and the success of treatment depends very much on reaching an effective drug concentration inside the cancerous tissue. Cancer cells can avoid the drug accumulation by having an active mechanism that expels the drugs from the cell.[1,2] The extrusion of the drug severely reduces the efficiency of treatment as tumors achieve high drug tolerance and survival rates. Due to this mechanism, cancer cells become protected against a broad variety of widely used anticancer agents such as doxorubicin, vincristine, methotrexate and many others.[3–5] The phenomenon has been branded multidrug resistance (MDR), and has been implicated in many cancers, including multiple myeloma, lymphoma and leukemia. Numerous considerations are factored in the appearance of an overall drug-resistant phenotype. These may include the origin of the cancerous tissue, the individual physiological response to drugs in terms of absorption, metabolism and delivery, and also genetic factors that affect resistance.[6] In the last two decades, several proteins have been identified as chief perpetrators of MDR in cancer, i.e., the P-glycoprotein (P-gp), MRP1/ABCC1, MRP2 and MRP3 proteins.[7] These proteins are...
transmembrane proteins, members of the ATP-binding cassette (ABC) transporter superfamily, and utilize ATP hydrolysis to extrude the substances outside of the cell. In the generally accepted alternating-access mechanism, the protein alternates between two configurations, inward- and outward-facing states.\[13\]

The subject of this study is human MRP1, a transmembrane protein of 1,531 amino acids capable of extruding a wide variety of anticancer drugs. The protein is typically expressed in all tissues of the human body (located primarily on the plasma membrane), but in some tissues (lungs, testes, kidneys, heart and placenta) its expression is significantly higher. It has been suggested that the biological role of MRP1 is related to detoxification mechanisms and its wide range of substrates allows it to handle many types of harmful substances.\[3\]

Elucidating the mechanism of the drug translocation is cardinal in designing drugs that will suppress the MRP1 clearance pathway. In contrast to the mouse P-gp transporter whose structure has been solved by Chang et al.,\[9\] the study of the human protein MRP1\[4,10,11\] is still limited to low-resolution methods.\[12,13\]

The MRP1 protein is made up of three transmembrane domains (TMDs): TMD0, TMD1 and TMD2. The first consists of five transmembrane helices (TMHs), while the other two each consist of six TMHs and one nucleotide-binding domain (NBD). It has been shown that TMD0 is not essential for transporter activity, but is related to the proper subcellular distribution of MRP1.\[14\]

The mechanism of action of MRP1 is rather complex; it includes the binding of the drug at the TMD in its inward orientation, the binding of ATP at the NBD site with subsequent structural conformation changes, transmission of the mechanical stress to the TMD forcing it to assume the outward-facing conformation, the release of the drug to the extracellular space and, finally, the relaxation of the system while releasing the ADP and phosphate from the NBD.\[3\]

In a recent publication, Jara and co-workers used fluorescence measurements and molecular dynamics to search for the drug binding site. Their observations indicated that the homology model of human MRP1 could readily predict the location of the binding site, identified on the 4th, 5th and 6th TMHs.\[15\]

DeGorter and co-workers proposed a homology model of MRP1\[16\] based on the outward-facing crystal structure of the bacterial ABC transporter SAV1866,\[17\] which does not include TMD0. In their work, three MRP1 mutants were studied focusing on a specific tyrosine residue (Y324) located on TMH6 of the transmembrane domain. This residue was replaced by either phenylalanine (Y324F), tryptophan (Y324W), or alanine (Y324A), and the transport activity was monitored using four different drugs. Two of the mutants (Y324W and Y324A) displayed, essentially, a very similar activity to the wild-type (Wt) protein. However, the Y324F mutant exhibited an enhanced reactivity (~150 %) with the four tested drugs.\[16\]

This observation is somewhat baffling, as both residues are quite similar in their properties. It should be mentioned that of the many tested mutations,\[16,18–20\] Y324F is one of the few that increases the activity of the protein.

Recently, Cole and a co-worker studied the function of five mutant proteins containing mutations that were predicted to affect the MRP1 activity. Of the five mutations that were tested, only those located on the TMD were able to modify the selectivity of the protein. This is a clear indication that the site where the affinity to the drug can be modulated is at the TMD section, not on the cytoplasmic-facing domain. Apparently, once we wish to evaluate the interaction of the protein with drugs the simulations can be limited to the TMD section.\[21\]

In the present study, we used the MRP1 homology model proposed by DeGorter et al. to investigate by molecular dynamics (MD) their intriguing observation that certain mutations at the TMD can accelerate the clearance rate of four different drugs.\[16\] Such an observation implies that in the Wt state there are some reaction steps, at the TMD sites, whose rates are comparable with the rate-limiting step of events taking place in the NBD section of the whole protein. Thus, partial acceleration of these steps will shorten the overall turnover time of the energy-driven drug translocation. This peculiar property is presently investigated by MD simulations on the TMDs, since they are directly involved in the drug-release step. To demonstrate that the cause of the accelerated clearance is indeed embedded in the structure of the TMD, we deliberately decoupled the TMD from the NBD, limiting the simulations to the structure of the TMD section. Based on our MD simulations, which corroborate the experimental data,\[16\] we offer a possible explanation for the working mechanism of MRP1, where Y324 serves as a hinge to the stable conformation of the outward-bound state of MRP1. Thus, our simulations provide a refined model that was achieved following relaxation of the protein in its physiological environment.

2 Experimental Section

2.1 MRP1 Homology Model

All simulations were based on the MRP1 model published by DeGorter et al. (modeled after the multiple alignment of SAV1866 bacterial transporter).\[16,17\] Before initiation of the project we had generated a new homology model of the MRP1 protein which turned out to be essentially identical with that of DeGorter. Accordingly, to be consistent with the already published model, we used the one of DeGorter in the present study.

The homology model depicts TMD1 and TMD2 of MRP1 in an outward-bound state (Figure 1). In this state, the protein is assumed to expose the translocation pathway space towards the extracellular matrix, while the substrate is in the low-affinity phase, ready to dissolve in the

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To clarify whether the modulation of the overall rate is a direct reflection of the structural rearrangement caused by the Y324 mutants, the simulations were limited to just the TMD section. The truncated protein was embedded in a pre-equilibrated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer, scaled up from the initial 128 lipid molecules to 250 in order to accommodate the large MRP1 protein. The bilayer membrane system was solvated using the SPC water model and further equilibrated for 10 ns. To ensure that a one-step relaxation process of the protein does not cause an artifact constriction of the transmembrane cavity, we additionally performed the solvation with an alternative procedure as a technical control. In this procedure, the restraints were released by five gradual steps each of 400 ps length. The results of the two solvation methods were practically identical, and thus we set forth with performing MD simulations for the Wt and the three mutants. Hence, our observations regarding the simulations reflect the internal dynamics of the protein and do not stem from the solvation process. In both cases the water molecules spontaneously penetrated the cavity of the channel and the number of solvent molecules was independent of the mode of relaxation. The total charge of the system was first neutralized by adding counterions. The ionic strength of the solution was raised to 0.1 M by adding Na\(^+\) and Cl\(^-\) ions at random locations. It should be noted that we did not observe entry of an ion to the transmembrane cavity in any of the simulations.

### 2.2 MD Details

The in silico removal of the NBDs split the protein into six chains and created artificial termini, which had to be stabilized so that the transmembrane helices would not gain artificial degrees of freedom that would be uncommon for the natural protein (Table 1). To avoid the rupture of the protein at the artificial termini, a restraining potential coefficient of 1000 kJmol\(^{-1}\)nm\(^{-2}\) was applied on the backbone atoms of the three end residues of each artificial terminus. Under this procedure the side chains of these residues could exercise some structural fluctuation. The list of residues in each TMH, the artificial termini and the restrained residues are detailed in Table 1 and Figure 1. The ionization states of the polar residues located in the TMHs were in accordance with their standard pK\(_a\) values and the physiological pH. The whole protein is designated as hMRP1 for the homology MRP1, and is presented in Figure 1.

![Figure 1. Representation of homology model of MRP1 in an outward-bound state. Purple: the region of the NBD domains that was truncated from the hMRP1 structure. Yellow: the restrained residues (three in each helix) used to prevent the structure from expanding. Blue: the unrestrained residues consisting of the TMHs and the loops that link them together. Orange: VDW representation of Y324 residue. The membrane is perpendicular to the z-axis. Two termini were left unrestrained due to being natural termini of the protein.](image)

<table>
<thead>
<tr>
<th>Chain</th>
<th>Chain residues</th>
<th>TMHs(^{[a]})</th>
<th>Residues subjected to restrained backbones(^{[b]})</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>L300–K396</td>
<td>6–7</td>
<td>R394–K396</td>
</tr>
<tr>
<td>B</td>
<td>D430–M495</td>
<td>8–9</td>
<td>D430–Q432, A493–M495</td>
</tr>
<tr>
<td>C</td>
<td>L536–K614</td>
<td>10–11</td>
<td>L536–V538, S612–K614</td>
</tr>
<tr>
<td>D</td>
<td>C971–S1045</td>
<td>12–13</td>
<td>L1043–S1045</td>
</tr>
<tr>
<td>F</td>
<td>Q1186–E1262</td>
<td>16–17</td>
<td>Q1186–A1188, A1260–E1262</td>
</tr>
</tbody>
</table>

\(^{[a]}\) The TMHs of MRP1 are comprised of the residues listed under the “Chain residues” column. \(^{[b]}\) The new termini created by the truncation process. The backbones of these residues were restrained to limit the additional degrees of freedom created by the truncation. The capping of the truncated residues, meaning all the non-natural termini, was set to be neutral (–COOH and –NH\(_2\)).
the short MRP1, where the NBD domains were truncated, as shMRP1.

The simulations were conducted using version 4.0.7 of the GROMACS package,[27–30] employing an extended version of the GROMOS53a6 force field,[31] which included Berger lipid parameters.[32] All simulations were conducted using the LINCS algorithm[33] to constrain bond lengths and angles of hydrogen atoms, thus allowing a time step of 2 fs. The system was subjected to energy minimization using the steepest descent algorithm and a tolerance of 1000 kJ mol⁻¹ nm⁻¹.

Simulations were run using velocity-rescale temperature coupling[34] at 310 K, employing a coupling constant of \( \tau_T = 0.1 \) ps. Pressure was kept constant by applying semi-isotropic coupling at 1 bar with a coupling constant of \( \tau_P = 1 \) ps, differentiating the z-axis (the membrane normal), using the Parrinello–Rahman barostat. A cutoff of 1.2 nm was used for van der Waals interactions, while long-range electrostatic interactions were computed using the PME algorithm.[37]

2.3 Counting the Water Molecules in the Translocation Pathway and Their Rate of Exchange with the Bulk

The analysis was based on the eight largest clusters of each trajectory. At first, each cluster was stripped to include only backbone coordinates and fitted into a cylinder-like shape to derive its average radius. This cylinder was then truncated by 1.2 nm on both sides, to exclude water molecules located at the orifice. With the remaining space, the number of water molecules was counted for each snapshot (Figure S1 in the Supporting Information). To calculate the rate of water flux along the length of the translocation pathway, molecules that resided inside the protein for less than 30 ps were excluded from the count.

2.4 Generation of Mutant MRP1 Proteins

The three mutations investigated by DeGorter et al.[16] were computationally generated using the WhatIf server,[38,39] with shMRP1 as template. The Y324 residue was replaced by alanine, tryptophan, or phenylalanine, and the resulting structures were geometrically optimized in such a way that the new residues did not clash or disrupt the overall structure of the protein. Each mutant was subjected to energy minimization, position-restrained equilibration and production runs.

2.5 Acquisition of Data

The simulations were carried out for 30 ns and snapshots were saved every 2 ps (15,000 frames). Counting of water molecules was executed using Beckstein's g_count package[40] The possible paths of the water-filled spaces crossing the membrane were calculated by the Travel Distance Suite CHUNNEL package.[41–44] The protein surface was probed for places that may sterically allow binding of water and their shape and volume was mapped, as comprehensively described in detail in reference [41]. In short, a putative travel depth was defined by assuming closed triangulated surfaces. Then, the travel path was calculated by mapping the protein and its surrounding solvent onto a grid, classifying the grid points to check whether they lie inside or outside a pre-defined convex hull, and then allocating the travel depth to the grid points. Next, each surface point was assigned a depth based on the grid cube it was located in, resulting in a computed travel depth for each point on the surface. Finally, the triangulated molecular surface, along with travel depth values that corresponded to each point of the surface based on travel depth, were read and visualized.

3 Results and Discussion

3.1 The Relaxation of the shMRP1 Structure

The relaxation dynamics of the shMRP1 under permanent and gradual position restraint release are presented in Figure 2. Once the protein is released and allowed to

![Figure 2](https://example.com/image.png)

**Figure 2.** The relaxation dynamics of the Wt shMRP1 embedded in the POPC membrane. (A) The increment of the protein’s RMSD for its backbone atoms. (B) The solvation dynamics of the transmembrane cavity. The black curves relate to the solvation of the shMRP1 for 2 ns under permanent position restraint of its heavy atoms. The alternative procedure, reducing the restraints by five decrements, each one of 400 ps long, is represented by the red curves.
react with the solvent under physiological conditions, its state is relaxed and subsequent increase of its RMSD backbone atoms is detected (Figure 2A). In the first case, when the protein was solvated for 2 ns with permanent position restraint on its heavy atoms, the water molecules diffused into the cavity, filling it with up to $\sim 260$ molecules (Figure 2B, black curve).

When the position restraint was removed in a single step, two processes occurred: the RMSD of the protein increased to a steady level of $\sim 0.5$ nm (Figure 2A, black curve), and in parallel the water content decreased to $\sim 50\%$ of the initial level (Figure 2B, black curve). The alternative procedure (Figure 2, red curves) was to reduce the restraints in five decrements, each one of 400 ps in length and releasing the force by 200 kJmol$^{-1}$nm$^{-2}$, with subsequent very small increase of the RMSD. Once the last restraint had been removed, both the RMSD and the water content increased to a stable level. Figure 3 depicts the decline of the water content of the three mutations together with the Wt (for clarity only a single trajectory for each system is presented). Apparently, the shMRP1 structure, when embedded in the membrane in the presence of a physiological solution, prefers a more condensed state than the homolog structure that was proposed, assuming structures with constriction of the aqueous space. In contrast, the mutant that exhibited a higher drug clearance capacity, Y324F, retained (in both simulations) a constant volume of the translocation pathway, in accordance with its higher transport activity of various drugs.

### 3.2 Characterization of the Simulations

Four sets of simulations were carried out, corresponding to the Wt protein and the three mutants, Y324F, Y324W and Y324A. For each set, each simulation started from exactly the same initial coordinates but with different random seeds. The numbers of the simulations for each initial structure are detailed in Table 2.

Once the shMRP1 structures were released from the constraints applied during the solvation phase, the protein experienced a gradual constriction lasting some 5–10 ns. This is best demonstrated by following the number of water molecules that accommodated the translocation pathway. In the case of the Wt simulations (Figure 3A), the water content declined from $180 \pm 15$ (at $t = 0$) to $100 \pm 20$ molecules. This decline had a clear dynamic trend progressing rather smoothly, over $\sim 7–10$ ns, towards a stable level. Figure 3B depicts the decline of the water content of the three mutations together with the Wt (for clarity only a single trajectory for each system is presented).

**Figure 3.** The variation in the number of water molecules inside the transporter transmembrane cavity as a function of simulation time. (A) Four different simulations of the Wt protein. (B) Wt1 and three simulations of the mutants, as explained in the text.
last 20 ns of the simulation time. However, the Y324F mutant exhibits 1200–1500 such events, which is some 3–5 times more than the value calculated for the Wt protein, ~8 times more than for the Y324W mutant, and ~4 times more than for the Y324A mutant (Table 3). These variations suggest that the dynamics of the water in the translocation pathway is tightly coupled with the intimate composition of the residues lining the inner space of the protein.

Table 3. The flux of water molecules across the transporter cavity as measured for the wild-type and the mutants.

<table>
<thead>
<tr>
<th>Simulation</th>
<th>Flux of water molecules[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt1</td>
<td>325</td>
</tr>
<tr>
<td>Wt2</td>
<td>127</td>
</tr>
<tr>
<td>Wt3</td>
<td>414</td>
</tr>
<tr>
<td>Wt4</td>
<td>434</td>
</tr>
<tr>
<td>Y324F1</td>
<td>1471</td>
</tr>
<tr>
<td>Y324F2</td>
<td>1195</td>
</tr>
<tr>
<td>Y324W1</td>
<td>181</td>
</tr>
<tr>
<td>Y324W2</td>
<td>139</td>
</tr>
<tr>
<td>Y324A1</td>
<td>154</td>
</tr>
<tr>
<td>Y324A2</td>
<td>519</td>
</tr>
</tbody>
</table>

[^a]: The flux was calculated for the last 20 ns of the trajectory (see text).

3.3 Cluster Analysis

The trajectories generated for the Wt and the mutated proteins were subjected to cluster analysis, looking for the representative structures.[^45] To focus on the properties of the well-solvated equilibrium state of the protein, the first 5 ns were treated as an equilibration period and the analysis was applied to the last 25 ns of the trajectories using the gromos algorithm[^29,46] with a conservative cutoff of 0.12 nm. The distribution of the clusters according to their size is given in Figure 4. The first 18 clusters comprise more than 98% of the total population of the structures sampled by the protein during the 25 ns simulation time. Each of the residual clusters contains less than 0.1% of the total population.

The distribution of the cluster size, as presented in Figure 4, indicates that the protein assumed multiple configurations that differ in energy by only a small increment. The relative size of the clusters appears to decrease as a smooth function where the ratio between consecutive clusters is smaller than twofold. Assuming that this ratio is an approximation of the equilibrium distribution between the clusters, the difference in the free energy between adjacent clusters will be ~1 $k_B T$ or even less than that. To visualize the difference between the shapes of the structures in the cluster, Figure 5 depicts a superposition of the eight largest ones, accounting for at least 85% of the last 25 ns that were used for the cluster analysis. The figure indicates that for each of the four shMRP1 proteins (Wt and three mutants), the largest clusters retain high similarity of the helices and the main differences (also accounting for most of the RMSD) are due to higher freedom of the loops. Representative structures of the largest cluster, after the 30 ns simulation, of Wt superimposed on each of the mutants are presented in Figure S2 in the Supporting Information.

While each protein exhibits small structural fluctuations, the comparison of the superposition clusters clearly

![Figure 4. Cluster analysis. The relative size of the clusters as calculated for the Wt and the Y324F, Y324W and Y324A mutations of the shMRP1 embedded in the POPC membrane. Cluster distribution amongst the various simulations. The clusters were calculated using the gromos algorithm with a 0.12 nm cutoff value.](image-url)
emphasizes the unique shape of the Y324F mutant; it is the only structure that retains the V shape of the translocation pathway. All other structures, including the Wt, experience a spontaneous constriction,[16] where the width of the EC opening is significantly smaller with respect to the homology model. The persistence of the constriction during the simulation suggests that it may impose a rate-limiting step on the overall catalytic cycle. The fact that the Y324F transporter has a higher clearance indicates that the other elements of the catalytic cycle are not limiting the velocity of the cycle.

3.4 The Putative Translocation Path

The putative path through which the drug is transported was calculated using the CHUNNEL program package. The analysis was applied on the structure of the largest clusters of the Wt and the Y324F simulations. Figure 6 depicts the projected translocation pathway as a series of spheres that transverse the full length of the protein. Figure 6A is for the Wt protein, while Figure 6B depicts the pathway across the Y324F mutant which, for the sake of clarity, was rotated by 180°. A similar pathway shape was recently reported for the EmrD protein. [47]

The analyses identify several water-filled pockets over the whole protein, yet in the figure we present only those that are merged into a continuous path extending from the IC to the EC side (Figure 6). On the cytoplasmic side there are two ports of entry that merge after ~1 nm forming the conducting channel. Our finding of two ports may well agree with experimental observations regarding passage of certain ligands from the intracellular side to the extracellular side of the protein. It was found that certain point mutations allowed transport of specific known ligands while the transport of others was blocked. For example, based upon mutation analysis, Cole et al. reported that two of the best characterized substrates of MRP1, the physiological metabolites leukotriene C4 (LTC4) and 17β-estradiol 17-β-D-glucuronide (E217G) exhibit different binding modes to the intracellular side of MRP1. This experimental data is in line with our putative translocation path calculations, and may hint that some ligands bind to and enter the protein through one port and others through the other port, and consequently certain point mutations hinder the passage of specific ligands but not others.[18–20] Still, considering that the structure under study is a truncated one, further elaboration on the shape of the path on the IC side is needed.

On the channel section between the merging point and the extracellular space the shape of the translocation path varies between the Wt and the Y324F mutant. The Wt protein exhibits a constriction (Figure 6A), where the passage seems to lose its continuity (see the red-colored marking in the figure); this is very similar to the constriction reported by Baker and co-workers for the Escherichia coli EmrD protein. [47] The constriction zone does not seal water efflux, as in the less frequent clusters of the Wt shMRP1 there is a continuous cavity that spans the full length of the protein. The constriction has temporal fluctuations, which, upon time averaging, functions as a bottleneck of the efflux process. The simulated structure of Y324F exhibits, at the site where the Wt is constricted, a well-expanded translocation pathway (for better visualization of the difference in the constriction zone, the structure of Y324F in Figure 6B was rotated by 180° in relation to the Wt protein). Apparently, the properties of the Y324 moiety are responsible for the formation of the bottleneck; thus, the specific replacement of Y324, which can participate in hydrogen bonding, by a more hydrophobic residue, prevents the inner space from collapsing.

3.5 Structure-Stabilizing Interactions

The key role of Y324 in forming the shape of the cross-membrane section of MRP1 was evaluated by calculating the energy of its interaction with the nearby environment. Figure 7 presents the Coulomb and Lennard-Jones poten-
tials of residue 324 (either the Wt tyrosine or the mutated phenylalanine), as calculated for the interaction with nearby residues or with the solvent. The upper panel depicts the energies of the Wt TMD, while the lower panel depicts the results of the Y324F mutated protein.

The Lennard-Jones interactions between the residues at position 324 and the solvent are rather weak and will not be discussed. Similarly, the Lennard-Jones interactions of residue 324 with the nearby amino acids are on the order of ~70 kJ/mol for the Wt and the mutant, and exhibit no significant differences between them.

In contrast to the Lennard-Jones interactions that are comparable for the Wt protein and the mutant, the nature of the electrostatic potentials is grossly different. At the initiation of the Wt simulations, Y324 maintained electrostatic interactions (ca. ~80 kJ/mol) with both the nearby residues and the water in the cavity. Once the simulation was initiated there were sequential mirror-image fluctuations: whenever the tyrosine moiety enhanced its electrostatic interaction with nearby residues (Figure 7, upper panel, blue trace), there was a parallel decrement in its solvation energy (green trace). The magnitude of these fluctuations was ~50 kJ/mol. Apparently, when the tyrosine is facing some nearby moiety it reduces its interaction with the solvent. The replacement of the tyrosine at position 324 by the nonpolar phenylalanine (or by tryptophan; see Figure S3 in the Supporting Information) grossly alters the pattern of stabilizing forces at the same domain (Figure 7, lower panel). The phenylalanine moiety maintains electrostatic interaction through the backbone atoms with its polar neighbors, although this interaction becomes gradually less favorable after ~20 ns (blue trace). This is because the phenylalanine slightly detaches from its neighboring residues, and interacts with the solvent. This is reflected by a moderate decrease until a relative stabilization in its solvation energy (green trace).

**Figure 7.** Energy calculations. Energy profiles of position 324 in the Wt (upper panel) and Y324F (lower panel) simulations. The energies calculated are electrostatic (Coulomb) and van der Waals (LJ) with neighboring residues and with the solvent.
These interactions were stable in intensity during the length of the simulation times. The lack of structural fluctuations in the vicinity of F324 rendered the system more stable, and no constriction events were noted.

The swapping of electrostatic partners, as exhibited by Y324, is readily accounted for by inspection of the orientation of Y324 (located on helix 6) with respect to the histidine moieties positioned on helix 7 nearby. Figure 8 depicts the distances of the Y324 residue from H382 (black line) and H386 (blue line) through the entire simulation. For the greater part of the simulation time, the distance between Y324 and H382 is very short and fluctuates between 0.2 and 0.3 nm, indicating the existence of reasonable contact. Moreover, it seems that Y324 can be at contact distance, most of the time, with both histidine residues H382 and H386 simultaneously, and for a while to be slightly closer to one than the other. As long as these contacts are stable, helices 6 and 7 keep a close distance between them, stabilizing the constricted shape of the channel.

3.6 A Cluster of Phenylalanine Residues Dictates the Cavity Characteristics

DeGorter et al.\[16\] tested the effect of the mutation at position 324 on the clearance of four different drugs. Of the four mutants, only one mutant (Y324F) exhibited, in all cases, a massive increase in the extrusion rate well above that of the Wt protein. Based on these results, and the homology model, DeGorter suggested that despite the apparent orientation of Y324 towards the cavity, its role is related to the structure–function relationship of the protein rather than to a direct interaction with substrates. The present simulations corroborate these predictions; the effect of the mutation is on the shape of the translocation pathway, which is common to all drugs.

Figure 9A depicts the representative center structure of the largest cluster (the structure with the highest number of neighbors was taken as the center of the cluster) of the Wt protein. Figure 9B represents the Y324F mutated protein. As seen in Figure 9A, and detailed in the lower frame, once the protein had assumed its more stable configuration in the membrane, the side chain of the tyrosine had rotated to form hydrogen bonds between its hydroxyl and the nitrogen atoms of the histidine residues, H382 and H386, located on helix 7. Specifically, the carbonyl oxygen of H382 forms a hydrogen bond with the hydroxyl hydrogen of Y324, whereas the hydrogen of the NH group of H386 interacts with the hydroxyl oxygen of Y324. This interhelical hydrogen bond network causes a contraction of the translocation pathway, with a subsequent effect on the ability of the drugs to diffuse toward the EC bulk.

In the Y324F mutant (Figure 9B), the phenylalanine residue interacts with its immediate aromatic neighbors in helix 6, F321 and F325, thereby preventing the attachment between helix 6 and helix 7. The lack of interaction between TMH6 and TMH7 allows the translocation pathway to maintain a funnel shape, which accounts for the higher flux of drug through the protein. Moreover, the packing of the three phenylalanine residues, F321, F324 and F325, twists TMH6 to form a kink, with subsequent rearrangement of another cluster of three phenylalanine residues (F329, F330, F331) located on the extracellular...
side of the protein. As a result, these three residues adopt different orientations, where their aromatic rings interact with the hydrocarbon chains of the phospholipids, enabling the positive charge of the nearby K332 residue to react with the negatively charged phosphate head group of POPC. Apparently, these forces are strong enough to fix the protein in a wide-open configuration, which accounts for the activity of the mutant.

Apparently, the subtle changes in the binding partners of residue 324 have a dramatic effect on TMH6; due to the Y324 residue, TMH6 gains flexibility and may stretch across the cavity (Figure 10A). This seems to be the molecular mechanism that causes the Wt cavity to tighten, making it narrower. In contrast, the Y324F mutation creates a relatively stable cluster of phenylalanine residues that forces a different orientation of TMH6; one part of it is found parallel to the \( z \)-axis, whereas the other extends away from the cavity (Figure 10B). Residue 324 affects tremendously the network of interaction of the nearby amino acids, driving them either to twist toward the protein residues (as for the Wt), or toward the lipid bilayer and establish a different network of residue interactions (as for the Y324F mutant). Hence, the movement of TMH6 seems to be regulating the size of the cavity of MRP1. The direction of this helix, crossing the cavity to the opposite side extending away from the cavity, might be a crucial event for the mechanism by which cavity size is adjusted in MRP1 in various stages of drug extrusion.

3.7 The Catalytic Cycle of MRP1

The catalytic cycle of MRP1 includes the binding of both ATP and the transposed drug, the conformational changes coupled with the transmittance of mechanical stress between the NBD and the TMD elements, and the final release of the substrate and hydrolysis products. The overall time constant of a catalytic cycle is the summation of the time constants of the non-overlapping partial reactions, as denoted in Equation 1.

\[
\tau = \Sigma k_{i}^{-1}
\]  

Thus, the rate of the overall reaction (\( \tau \)) will be slower than any partial reaction, including that of the rate-limiting step. The observation of DeGorter\[14\] that the overall flux had increased (in the case of Y324F) by 150% implies that drug clearance from the cavity of the outward-facing configuration is sufficiently slow to contribute significantly to the overall length of the catalytic cycle. As the translocation path is stabilized in a constantly open configuration, the rate of clearance is enhanced with substantial acceleration of the catalytic cycle.

4 Conclusions

In the present study, we isolated the TMD of MRP1 from its ATP-consuming functional element (NBD) and inves-
igated whether the structural changes innate to the specific replacement of tyrosine by phenylalanine suffice to account for the overall increase in the rate of catalysis.

The simulations of the translocation pathway structure revealed that once the structure is allowed to accommodate for the new environment (membrane plus solvent and physiological ionic strength) there is a well-defined relaxation of the structure to form a more condensed structure that is stabilized by hydrogen bonds between helices 6 and 7. The motion of the helices located inside the core of the TMD is followed by some deformation of helices 10 and 12, coupled with relative translation–rotation of the other helices, leading to a decrement of the water-accessible space within the translocation pathway. We consider this event as the mechanism that slows the rate at which a drug molecule can diffuse from the IC side of the TMD towards the EC orifice of the protein. The constricted state is in equilibrium with less tight structures, thus forming a “fluctuating bottleneck” that slows the exit of the drug yet does not block it. The replacement of the tyrosine by phenylalanine, which fails to establish the hydrogen bond between helices 6 and 7, allows the protein to form a set of hydrophobic interactions that arrest helix 6 in a rigid form that keeps the translocation pathway clear and open. In this state, the bottleneck configuration cannot be formed and the flux out of the TMD to the EC bulk is unhindered.

Our study shows that simulations of the homology model provide atomic resolution insights that account for the WT and specific mutants of MRP1 as observed by experimental data. In particular, the Y324F mutant is characterized by a continuous wide translocation pathway and this may explain why it extrudes drugs better than its WT counterpart. The compatibility substantiates the homology structure as a working model of the protein after being processed on the website: www.gromacs.org.

Abbreviations: ABC: ATP-binding cassette; MD: molecular dynamics; MDR: multidrug resistance; MRP1: multidrug resistance protein 1; NBD: nucleotide-binding domain; POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; shMRP1: short homology MRP1; TMD: transmembrane domain; TMH: transmembrane helix.

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References


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