JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

COMMUNICATION

pubs.acs.org/JACS

Effect of the F610A Mutation on Substrate Extrusion in the AcrB Transporter: Explanation and Rationale by Molecular Dynamics Simulations

Attilio V. Vargiu,[*,†] Francesca Collu,[†,‡,¶] Robert Schulz,[§] Klaas M. Pos,[∥] Martin Zacharias,[⊥] Ulrich Kleinekathöfer,[§] and Paolo Ruggerone[*,‡]

[†]CNR-IOM, Unità SLACS, S.P. Monserrato-Sestu Km 0.700, I-09042 Monserrato (CA), Italy

[‡]Department of Physics, University of Cagliari, S.P. Monserrato-Sestu Km 0.700, I-09042 Monserrato (CA), Italy

[§]School of Engineering and Science, Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany

[∥]Cluster of Excellence Frankfurt - Macromolecular Complexes and Institute of Biochemistry, Goethe University Frankfurt, Max-von-Laue-Str.9, D-60438 Frankfurt am Main, Germany

[⊥]Physik-Department, Technische Universität München, James-Franck-Str. 1, D-85748 Garching, Germany

Supporting Information

ABSTRACT: The tripartite efflux pump AcrAB-TolC is responsible for the intrinsic and acquired multidrug resistance in *Escherichia coli*. Its active part, the homotrimeric transporter AcrB, is in charge of the selective binding of substrates and energy transduction. The mutation F610A has been shown to significantly reduce the minimum inhibitory concentration of doxorubicin and many other substrates, although F610 does not appear to interact strongly with them. Biochemical study of transport kinetics in AcrB is not yet possible, except for some β-lactams, and other techniques should supply this important information. Therefore, in this work, we assess the impact of the F610A mutation on the functionality of AcrB by means of computational techniques, using doxorubicin as substrate. We found that the compound slides deeply inside the binding pocket after mutation, increasing the strength of the interaction. During subsequent conformational alterations of the transporter, doxorubicin was either not extruded from the binding site or displaced along a direction other than the one associated with extrusion. Our study indicates how subtle interactions determine the functionality of multidrug transporters, since decreased transport might not be simplistically correlated to decreased substrate binding affinity.

Multidrug resistance (MDR) mechanisms in bacteria render a large spectrum of chemically unrelated antibiotics ineffective and represent one of the most serious impediments to improved healthcare today.[1−8] The spread of MDR and the re-emerging of pathogens occur at a moment when no truly novel antibacterial compound is undergoing clinical trials, which means no new drugs will become available in the next six to eight years.[9−12] One of the main mechanisms of MDR is the active transport of drugs out of the cell through specific protein complexes called efflux pumps.[4,5,9,13−17]

MDR is of particular concern for Gram-negative bacteria comprising many human pathogens that are very difficult to treat.[13−15,18,19] Major efflux systems in these organisms consist of a tripartite assembly[4,20−24] of (a) an active transporter of the resistance-nodulation-division (RND) family, partially embedded in the inner membrane, which works as a drug-proton antiporter fueled by the transmembrane electrochemical gradient and is responsible for the drug specificity;[4,25,26] (b) a channel-like outer-membrane factor protruding into the periplasm;[27−30] and (c) an oligomer of a membrane-fusion protein, which links the two former components.[24,31]

AcrB of *Escherichia coli* is one of the best characterized RND transporters.[6,32] Its structure has been solved as an asymmetric homotrimer[33−35] in which each monomer adopts a different conformation (hereafter Loose (Access), Tight (Binding), and Open (Extrusion), or L, T, and O, respectively, following ref 34). Furthermore, Murakami et al.[33] solved the structures of AcrB with a doxorubicin or a minocyclin molecule located in a particular binding pocket in the porter domain of the T monomer. On the basis of this structural information and other biochemical data,[15,25,36−38] a *functional rotation* mechanism has been proposed[26,33,34] where each monomer neatly assumes the conformations L, T, or O. The L and/or T monomer allow the entrance of the substrate, which is accommodated in the binding pocket when the monomer is in the T conformation. Subsequently, the transition from T to O should pump the substrate from the binding pocket toward the TolC-docking domain.

Recent experimental[39] and computational[40,41] work investigated the putative path for substrates through the periplasmic porter domain and identified connections between the conformational transitions of the monomers and substrate displacement from the binding pocket.

In ref 40, a full atomistic model of AcrB in complex with doxorubicin was used in conjunction with targeted molecular dynamics (TMD) simulations[42] to mimic the conformational changes occurring on the protein along the T → O step of the functional rotation.

Using a similar approach and additional docking tools, we investigate here the effect of the *in silico* substitution F610A on

Received: March 24, 2011
Published: June 27, 2011

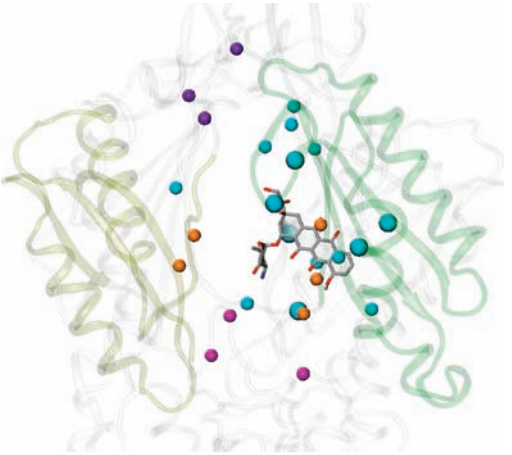

Figure 1. AcrB residues examined in this work in relation to the interaction with doxorubicin. The Tight (T) monomer of the protein is shown in cartoon representation in transparent gray with domains PC1 and PC2 in green and yellow, respectively. Doxorubicin (initial position in MUTANT) is depicted using sticks and colored accordingly to atom types (gray, red, and blue for carbon, oxygen, and nitrogen, respectively). The selections of the residues belonging to the putative extrusion pathway are colored according to ref 39, i.e., the orange, magenta, cyan, and violet beads refer to the *Cleft*, *Cleft*$_b$, the extended binding pocket, and the *Gate*, respectively. In addition, residues of the binding pocket interacting with doxorubicin as described in ref 33 are also shown as cyan spheres with a larger radius.

substrate binding and transport. This substitution in the binding pocket reduces the MIC of doxorubicin and many other AcrB substrates.[43] Interestingly, residue F610 interacts weakly with doxorubicin[33,40] and is not directly taking part in the zipper-like squeezing of the binding pocket described before.[40] In contrast, the substitution of other residues of the binding pocket (namely, F136, F178, F615, F617, F628) which are in direct contact with substrates and involved in drug displacement, has remarkably less impact on the efficiency of the efflux system.[43] To shed light on the molecular reasons behind the effects of the F610A substitution, we compare the binding pose and the displacement of doxorubicin along (several TMD simulations of) the T → O transition in the AcrB F610A variant with previous data on the wild-type protein.[40]

Hereafter, we refer to the wild-type and F610A variant of AcrB in complex with doxorubicin as WT and MUTANT, respectively. For the sake of clarity, we mainly discuss here two representative TMD trajectories that highlight different outcomes of the simulations, the remaining ones showing very similar trends (see Supporting Information for more details and for a critical discussion of the validity of our approach). The comparison will be done in terms of displacements from the binding pocket and interactions with it and with the gate leading toward the central funnel (residues Q124, Q125 and T758, hereafter *Gate*),[35] as well as the hydration of doxorubicin. In addition, we evaluated the interaction of the drug with the following residues:[39] F664, F666, L668, R717, and L828 (*Cleft*); D566, E673, and T676 (bottom of the *Cleft*, hereafter denoted as *Cleft*$_b$); S134, F136, Q176, F178, E273, N274, D276, I277, Y327, and F617 (extended binding pocket); Q124 and T758 (reduced *Gate*). These regions have been recently proposed to be part of the drug uptake and extrusion pathways.[39] All of those selections are indicated in Figure 1.

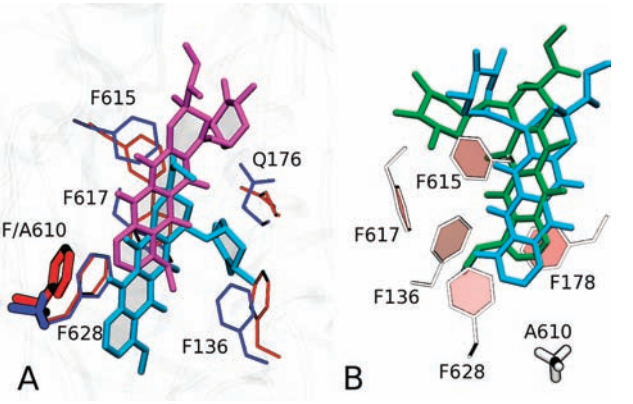

Figure 2. (A) Configurations of the drug within the binding pocket in WT and MUTANT after 5 ns MD equilibration. The ligand (colored cyan and magenta in MUTANT and WT, respectively) and residues of the binding pocket (colored blue and red in MUTANT and WT, respectively) are shown in sticks, while the T monomer is shown in the background in gray cartoon representation. Thicker sticks highlight the residue 610 in both systems. (B) Superimposition of doxorubicin molecules at the positions found at the end of the MD equilibration (blue sticks) or via docking calculations (green). The phenylalanine residues of the binding pocket are shown, as well as A610.

The first remarkable difference is found in the stable position of the drug within the binding pockets of WT and MUTANT. In MUTANT, doxorubicin slid down by ∼4.5 Å in the binding pocket between subdomains PC1 and PN2 (Figure 2), getting closer to the *Cleft* than in WT. Docking calculations using the ATTRACT[44] package, which includes ligand and receptor flexibility, have further validated these findings (see Supporting Information for details). As shown in Figure 3, a similar pose (rmsd of 2.4 Å with respect to the aforementioned one) resulted from the docking procedure. In addition, this position also yields the highest score in terms of binding energy and cluster population (data not shown). The interaction energy of the drug with residues of the binding pocket in MUTANT is larger by ∼10 kcal/mol as compared to WT (−23 vs −13 kcal/mol), and is essentially due to van der Waals interaction. This indicates a closer packing of the drug within the binding pocket of MUTANT with respect to WT, which is also consistent with the smaller number of water molecules within 3.5 Å of the drug (22 vs 30).

Figure 3A collects the displacements of the drug along T → O step in MUTANT (representative of one group of trajectories) and WT, relative to the binding pocket and to the *Gate*. In MUTANT, the ligand moves in a direction almost perpendicular to the one found in WT and does not approach the *Gate* but slightly retrogrades toward the *Cleft* (Figure 3B). Doxorubicin was located 3−4 Å closer to these regions than in WT already in the initial structures, and the difference increases up to ∼10 Å at the end of the simulation. This behavior was reproduced in additional simulations (see Figure S1). In a second group of simulations, the displacement of the drug was less pronounced (Figure S1). Thus, different events are possible during the T → O transition, but importantly, we *never* observed doxorubicin moving toward the *Gate* in MUTANT: either the ligand does not experience a large displacement or it moves in a direction almost perpendicular to the one detected in WT. Obviously, this does not exclude that doxorubicin could indeed move toward the gate, but the probability of such an event should be drastically reduced

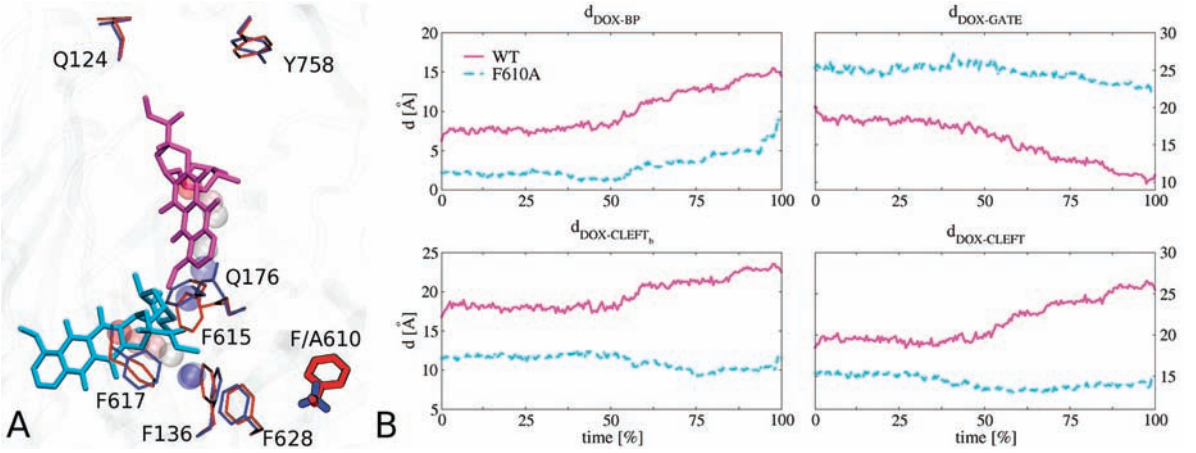

Figure 3. (A) The position of doxorubicin and the residues of the binding pocket for WT and MUTANT at the end of the T → O simulated functional rotation step. The color code is the same as in Figure 2A. The colored transparent beads represent the positions of the center of mass of the ligand from the beginning (blue) to the end (red) of the simulation. (B) Plot of the distances between the centers of mass of the ligand and the relevant selections of the protein vs simulation time.

according to our findings. The analysis of the interaction energies confirms that in MUTANT the drug maintains quite strong interactions with the binding pocket along the whole trajectory, but not with the *Gate* (Figures S2 and S3). This is in contrast to what is found in WT, where the interactions with the binding pocket residues are progressively lost, while those with the *Gate* increase with time (Figures S2 and S3). Finally, while no clear differences were observed between the WT and MUTANT concerning the interaction of the drug with the extended binding pocket (which has been defined considering the interaction of many different substrates with residues of the transporter,[39] while that defined in ref 33 is relative to doxorubicin and as such is more representative here), only in MUTANT the ligand interacts with the *Cleft*, which contains residues lining the entrance of a channel leading to the binding pocket.

A molecular mechanism can be proposed from our results for the observed stagnation of doxorubicin transport in the F610A variant. The long dwelling period of the substrate in the binding pocket of MUTANT, due to an enhanced interaction with its residues, could either block the binding of a second molecule during the next cycles of the functional rotation (see also Figure 4) or it could hinder crucial conformational changes of the protein. Consistent with this hypothesis is the persistence of doxorubicin at the binding pocket in 5 of the 7 simulations carried out for the T → O transition (Figure S1). Even in the case of a successful detachment from the binding pocket, doxorubicin moves in a direction almost perpendicular to that toward the *Gate* (Figure 3A). Furthermore, the ligand, which stays between the *Cleft* and the binding pocket, could interfere with the uptake of additional substrates by the L monomer (Figure 4).

Summarizing, we investigated the molecular mechanism behind the occurrence of reduced functionality due to the F610A substitution in the AcrB multidrug transporter, using a doxorubicin molecule as a probe. Our docking and MD simulations of the AcrB F610A variant in the LTO state show the presence of a slightly different binding pose of the ligand as compared to the wild-type protein. Site-directed mutation of residues proposed to be within the binding pocket is a popular method to confirm that a particular residue is indeed in the pocket and plays a functionally significant role. It is often assumed that the positive results,

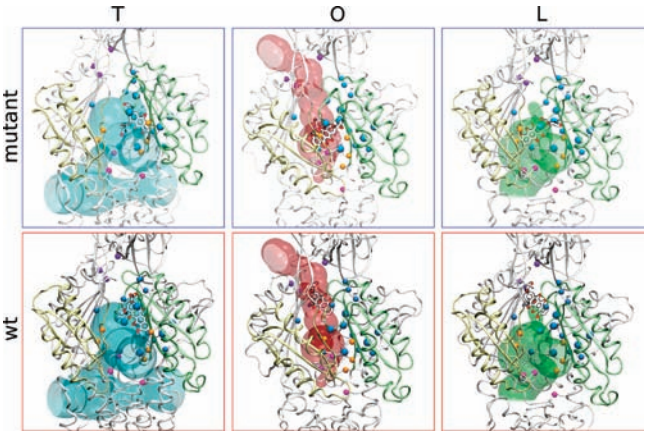

Figure 4. Displacement of doxorubicin compared to the morphology of the channels that are present in the three states (L, T, or O) of the doxorubicin-bound monomer. The upper and lower images (enclosed by blue and red boxes, respectively) represent conformations of MUTANT and WT, respectively, with panels from the left to the right referring to the conformations T, O, and L of the monomer. Protein and ligand are represented as in Figure 1, and channels determined with the CHUNNEL package[45] are shown by transparent surfaces (azure, red, and green refer to T, O, and L conformations, respectively). The calculation with CHUNNEL has been performed on the structures of the protein without ligand. In the initial conformation (left column), the ligand is located aside of a channel ending at the periplasmic entrance cleft. During the transition T → O in WT, doxorubicin reaches the upper part of the channel leading through the gate to the TolC-docking domain. In contrast, the ligand is found close to the Cleft leading to the periplasm in MUTANT (central column). In this position, it will most likely interfere with the uptake of additional substrates when the monomer will assume the L conformation along the functional rotation cycle (right column).

that is, the loss of activity in variants, is due to impaired binding of the substrate to the pocket. However, this is not always the case: we found a stronger interaction of the ligand with the binding pocket in the mutated protein, resulting in significant differences in the dynamics of the substrate along the T → O step of the functional rotation. The analysis of these differences

suggests a molecular-level picture of the mechanism of impaired drug export, which does not contrast with the data available in the literature and adds a piece of information about the functioning of AcrB transporter. A similar finding has been reported for the UapA purine transporter.[46] In terms of perspective, we note that our results on the F610A substitution suggest a general mechanism of AcrB inactivation, as the substitution has shown to affect a large number of substrates of AcrB. Preliminary results (data not shown) support our picture indicating that the F610A substitution largely affects even the binding pose of minocycline, while other substitution (e.g., F136A) do not induce a tight fit of doxorubicin within the pocket. Further TMD simulations with doxorubicin and minocycline in complex with the AcrB variants reported in ref 43 are being set up and will be reported elsewhere.

In conclusion, findings presented here could be a useful starting point to investigate the interaction of wild-type or mutant transporters at a molecular level with additional compounds belonging to different classes. Hopefully, it would be possible soon to pinpoint possible general hot spots and determinants affecting the functioning of the transporter.

■ ASSOCIATED CONTENT

Supporting Information. Methods, table, and graphs summarizing simulation data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

vargiu@dsf.unica.it; paolo.ruggerone@dsf.unica.it

Present Addresses

[¶]Department of Chemistry and Biochemistry, Universität Bern, Freiestrasse 3, CH-3012 Bern, Switzerland.

■ ACKNOWLEDGMENT

We are grateful to H. Nikaido for discussion and reading the manuscript and to J. Bohnert for stimulating discussions. M. Dessalvi is acknowledged for technical support. We acknowledge computer time from CINECA, Cybersar, CASPUR, and the DEISA project. This study was supported by the European Union, FP6 grant MRTN-CT-2005-019335 (Translocation) and by the COST Action BM0701 *ATENS*. A.V.V. acknowledges financial support from "Regione Autonoma della Sardegna" through a Research Fellow on fundings "PO Sardegna FSE 2007-2013, L.R.7/2007 Promozione della ricerca scientifica e dell'innovazione tecnologica in Sardegna". K.M.P. acknowledges the support of the Swiss National Foundation, the German Research Foundation (SFB 807, Transport and Communication across Biological Membranes) and DFG-EXC115 (Cluster of Excellence Macromolecular Complexes at the Goethe-University Frankfurt).

■ REFERENCES

(1) Schweizer, H. P. *Genet. Mol. Res.* **2003**, *2*, 48–62.
(2) Fischbach, M. A.; Walsh, C. T. *Science* **2009**, *325*, 1089–93.
(3) Blair, J. M.; Piddock, L. J. *Curr. Opin. Microbiol.* **2009**, *12*, 512–9.
(4) Higgins, C. F. *Nature* **2007**, *446*, 749–57.
(5) Alekshun, M. N.; Levy, S. B. *Cell* **2007**, *128*, 1037–50.
(6) Li, X. Z.; Nikaido, H. *Drugs* **2009**, *69*, 1555–623.
(7) Lomovskaya, O.; Zgurskaya, H. I.; Totrov, M.; Watkins, W. J. *Nat. Rev. Drug Discovery* **2007**, *6*, 56–65.
(8) Pages, J. M.; Amaral, L. *Biochim. Biophys. Acta* **2009**, *1794*, 826–33.
(9) Payne, D. J. *Science* **2008**, *321*, 1644–5.
(10) Barker, J. J. *Drug Discovery Today* **2006**, *11*, 391–404.
(11) Finch, R. *Lancet Infect. Dis.* **2010**, *10*, 754.
(12) Simmons, K. J.; Chopra, I.; Fishwick, C. W. G. *Nat. Rev. Microbiol.* **2010**, *8*, 501–10.
(13) Boucher, H. W.; Talbot, G. H.; Bradley, J. S.; Edwards, J. E.; Gilbert, D.; Rice, L. B.; Scheld, M.; Spellberg, B.; Bartlett, J. *Clin. Infect. Dis.* **2009**, *48*, 1–12.
(14) Poole, K. *J. Antimicrob. Chemother.* **2005**, *56*, 20–51.
(15) Nikaido, H.; Takatsuka, Y. *Biochim. Biophys. Acta* **2009**, *1794*, 769–81.
(16) Tal, N.; Schuldiner, S. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 9051–6.
(17) Brotz-Oesterhelt, H.; Sass, P. *Future Microbiol.* **2010**, *5*, 1553–79.
(18) Chevalier, J.; Mulfinger, C.; Garnotel, E.; Nicolas, P.; Davin-Régli, A.; Pagès, J.-M. *PLoS One* **2008**, *3*, e3203.
(19) Lautenbach, E. *J. Infect. Dis.* **2009**, *200*, 838–40.
(20) Touze, T.; Eswaran, J.; Bokma, E.; Koronakis, E.; Hughes, C.; Koronakis, V. *Mol. Microbiol.* **2004**, *53*, 697–706.
(21) Tikhonova, E. B.; Zgurskaya, H. I. *J. Biol. Chem.* **2004**, *279*, 32116–24.
(22) Nehme, D.; Poole, K. *J. Bacteriol.* **2007**, *189*, 6118–27.
(23) Pos, K. M. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 6893–4.
(24) Symmons, M. F.; Bokma, E.; Koronakis, E.; Hughes, C.; Koronakis, V. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 7173–8.
(25) Murakami, S. *Curr. Opin. Struct. Biol.* **2008**, *18*, 459–65.
(26) Pos, K. M. *Biochim. Biophys. Acta* **2009**, *1794*, 782–93.
(27) Koronakis, V.; Sharff, A.; Koronakis, E.; Luisi, B.; Hughes, C. *Nature* **2000**, *405*, 914–9.
(28) Higgins, M. K.; Eswaran, J.; Edwards, P.; Schertler, G. F. X.; Hughes, C.; Koronakis, V. *J. Mol. Biol.* **2004**, *342*, 697–702.
(29) Zgurskaya, H. I.; Yamada, Y.; Tikhonova, E. B.; Ge, Q.; Krishnamoorthy, G. *Biochim. Biophys. Acta* **2009**, *1794*, 794–807.
(30) Bavro, V. N.; Pietras, Z.; Furnham, N.; Pérez-Cano, L.; Fernández-Recio, J.; Pei, X. Y.; Misra, R.; Luisi, B. *Mol. Cell* **2008**, *30*, 114–21.
(31) Mikolosko, J.; Bobyk, K.; Zgurskaya, H. I.; Ghosh, P. *Structure* **2006**, *14*, 577–87.
(32) Li, X. Z.; Nikaido, H. *Drugs* **2004**, *64*, 159–204.
(33) Murakami, S.; Nakashima, R.; Yamashita, E.; Matsumoto, T.; Yamaguchi, A. *Nature* **2006**, *443*, 173–9.
(34) Seeger, M. A.; Schiefner, A.; Eicher, T.; Verrey, F.; Diederichs, K.; Pos, K. M. *Science* **2006**, *313*, 1295–8.
(35) Sennhauser, G.; Amstutz, P.; Briand, C.; Storchenegger, O.; Grütter, M. G. *PLoS Biol.* **2007**, *5*, e7.
(36) Takatsuka, Y.; Nikaido, H. *J. Bacteriol.* **2009**, *191*, 1729–37.
(37) Pietras, Z.; Bavro, V. N.; Furnham, N.; Pellegrini-Calace, M.; Milner-White, E. J.; Luisi, B. F. *Curr. Drug Targets* **2008**, *9*, 719–28.
(38) Seeger, M. A.; Von Ballmoos, C.; Eicher, T.; Brandstätter, L.; Verrey, F.; Diederichs, K.; Pos, K. M. *Nat. Struct. Mol. Biol.* **2008**, *15*, 199–205.
(39) Husain, F.; Nikaido, H. *Mol. Microbiol.* **2010**, *78*, 320–30.
(40) Schulz, R.; Vargiu, A. V.; Collu, F.; Kleinekathöfer, U.; Ruggerone, P. *PLoS Comput. Biol.* **2010**, *6*, e1000806.
(41) Yao, X. Q.; Kenzaki, H.; Murakami, S.; Takada, S. *Nat. Commun.* **2010**, *1*, 117.
(42) Schlitter, J.; Engels, M.; Kruger, P.; Jacoby, E.; Wollmer, A. *Mol. Simul.* **1993**, *10*, 291–308.
(43) Bohnert, J. A.; Schuster, S.; Seeger, M. A.; Fähnrich, E.; Pos, K. M.; Kern, W. V. *J. Bacteriol.* **2008**, *190*, 8225–9.
(44) Zacharias, M. *Protein Sci.* **2003**, *12*, 1271–82.
(45) Coleman, R. G.; Sharp, K. A. *Biophys. J.* **2009**, *96*, 632–45.
(46) Papageorgiou, I.; Gournas, C.; Vlanti, A.; Amillis, S.; Pantazopoulou, A.; Diallinas, G. *J. Mol. Biol.* **2008**, *382*, 1121–35.