Published in final edited form as:

Proteins. 2006 January 1; 62(1): 152–158. doi:10.1002/prot.20743.

Dynamics of the Trimeric AcrB Transporter Protein Inferred From a B-Factor Analysis of the Crystal Structure

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Abstract

The *Escherichia coli* AcrB multi-drug transporter recognizes a wide range of toxic chemicals and actively extrudes them from cells. The molecular basis of multidrug transport in AcrB remains unknown. Herein, we describe normal mode analyses to study important regions for drug recognition and extrusion in this transporter. Based on the X-ray structure of AcrB, an elastic network model has been able to correct errors arising from crystal imperfection in the experimental B-factors. The results allow us to understand the functional dynamics of this membrane protein. It is expected that this technique can be applied to other membrane proteins with known structures.

Keywords

multidrug transporter; efflux pump; B-factors; anisotropic network model (ANM); normal mode analysis; membrane protein

INTRODUCTION

The bacterial AcrB multidrug efflux transporter is a membrane protein that shows the widest substrate specificity among all known multidrug efflux pumps, ranging from most of the currently used antibiotics, disinfectants, dyes, detergents, to simple solvents.^{1,2} It has been previously reported that the multidrug binding site of AcrB is located at the upper half portion of an extremely large central cavity formed by the transmembrane (TM) domain of the pump.^{3,4} This binding presumably corresponds to the first step in the drug extrusion process. The complete picture of multidrug extrusion by AcrB, however, is not clear. Herein, we perform normal mode analysis to study important regions for drug recognition and extrusion in the AcrB efflux pump. It is expected that this technique can be applied to study the functional dynamics of other membrane proteins of known crystallographic structures.

AcrB is a member of the resistant-nodulation-division (RND) family transporters. This inner membrane efflux pump, AcrB, interacts with a periplasmic membrane fusion protein, AcrA,⁵ and an outer membrane channel, TolC,⁶ to mediate the extrusion of drugs across both membranes of *Escherichia coli*. The structural work from Yamaguchi's group⁷ revealed that the unliganded AcrB is a homotrimer. As shown in Figure 1, each subunit of AcrB contains

12 TM helices and two large periplasmic domains. The periplasmic domains of AcrB form a funnel-like structure at the top. This funnel region presumably interacts with the bottom of the periplasmic tunnel of the outer membrane channel protein TolC during drug extrusion.⁷ Below the funnel is a connected narrow pore that leads down to the large central cavity, with the diameter of 35 Å, formed by the TM domains of the three protomers of AcrB. At each protomer interface, there is a vestibule that opens the central cavity to the external surface of the lipid bilayer. These vestibules allow direct access of drugs from the periplasm as well as from the outer leaflet of the cytoplasmic membrane.⁷ It has been shown that four different ligands, ciprofloxacin, dequalinium, rhodamine 6G, and ethidium, bind at the wall of the upper portion of the central cavity.³ The periplasmic domain of the trimer also forms three prominent depressions facing the periplasm. These depressions have been suggested by Murakami et al.⁷ as possible interaction sites for the periplasmic linker protein AcrA.

In this article, we predict the AcrB multidrug binding sites as well as its important interaction sites by calculating theoretical B-factors of the structure of AcrB. To understand the mechanisms of drug recognition and extrusion by multidrug efflux pumps, knowledge about dynamical motions of the proteins is highly desirable. Usually, the B-factors obtained in protein crystallography contain valuable information about the dynamical behavior and the functional mechanism of a protein. Regions with large B-factors are usually more flexible and functionally important. However, one common problem with many membrane protein X-ray structures⁸ is that their overall B-factors are enormously high so that they cannot reflect the real functional dynamics of the proteins. The reason for these high B-factors is not yet clear. One possibility is that it is due to the difficulty of obtaining well-ordered crystals for membrane proteins. Large static disorder in the crystal lattice is manifested as large B-factors. The other possibility is that the high B-factors could be attributed to detergent micelles that enhance dynamical fluctuations in the protein crystal.

If the cause of the problem is static disorder due to poor crystal quality, the situation can be remedied by using computational modeling. B-factor patterns in a protein crystal structure are dominated by large-scale low-frequency motions of the system, and previous work for globular proteins showed that the system behaves like a continuum elastic media which can be modeled quite accurately with elastic network models as described in Methods.⁹⁻¹⁹ We use elastic network model calculations of the trimeric AcrB transporter protein crystal structure (1OY6) to study the dynamically active regions of the protein and the types of motion in these active regions. A possible mechanism for drug extrusion used by the pump is discussed based on the motions emerging from our analysis.

METHODS

The elastic network model⁹⁻¹⁹ has been demonstrated to be successful in representing the overall motions of globular proteins, and in particular the slowest motions derived from normal mode analysis. In this model, the nodes of the network are taken as the α -carbon sites of the residues and the linkers are harmonic springs between sufficiently close residue pairs. This simplest elastic network model is the Gaussian network model^{9-12,17} in which the fluctuations are assumed to be isotropic with no directional preferences.⁹ However, from detailed molecular dynamics simulations, it is known that the motions are not symmetric in all directions.²⁰ An extension of the Gaussian network model, called the anisotropic network model (ANM), incorporates the anisotropic effects on fluctuation dynamics,¹⁶ yielding the three-dimensional directions of the motion. The large-scale collective motions obtained from elastic network models are closely related to those extracted from atomistic molecular dynamics studies,²¹ which demonstrates the effectiveness of these coarse-grained models in the analysis of the structure-function relationships of proteins and their complexes.

In the ANM model, residues i and j with positions and are coupled with a spring potential

$$V_{ij} = \gamma/2 \left(\vec{s}_{ij} - \vec{s}_{ij}^0 \right)^2 = \gamma/2 \left(\Delta \vec{R}_i - \Delta \vec{R}_j \right)^2 \quad (1)$$

where $\vec{s}_{ij}$ and $\vec{s}_{ij}^0$ are the separation and equilibrium vectors between residues i and j . In the case of N residues, the second derivatives of the overall potential are expressed as a $3N \times 3N$ Hessian Matrix

$$H = \begin{pmatrix} H_{1,1} & H_{1,2} & \dots & H_{1,N} \\ H_{2,1} & H_{2,2} & \dots & H_{2,N} \\ \dots & \dots & \dots & \dots \\ H_{N,1} & H_{N,2} & \dots & H_{N,N} \end{pmatrix} \quad (2)$$

with

$$H_{ij} = \begin{pmatrix} \partial^2 V / \partial X_i \partial X_j & \partial^2 V / \partial X_i \partial Y_j & \partial^2 V / \partial X_i \partial Z_j \\ \partial^2 V / \partial Y_i \partial X_j & \partial^2 V / \partial Y_i \partial Y_j & \partial^2 V / \partial Y_i \partial Z_j \\ \partial^2 V / \partial Z_i \partial X_j & \partial^2 V / \partial Z_i \partial Y_j & \partial^2 V / \partial Z_i \partial Z_j \end{pmatrix} \quad (3)$$

In the harmonic model, fluctuations obey a Gaussian distribution, and the cross-correlation between residue fluctuations is given by

$$\begin{aligned} \langle \Delta \vec{R}_i \cdot \Delta \vec{R}_j \rangle &= (1/Z_N) \int \left(\Delta \vec{R}_i \cdot \Delta \vec{R}_j \right) \exp \left(-V/k_B T \right) d \left\{ \Delta \vec{R} \right\} \\ &= k_B T \cdot \text{tr} \left(H_{ij}^{-1} \right) \end{aligned} \quad (4)$$

The mean-square fluctuations of individual residues are

$$\begin{aligned} \langle \Delta \vec{R}_i \cdot \Delta \vec{R}_i \rangle &= k_B T \cdot \text{tr} \left(H_{ii}^{-1} \right) \\ &\approx k_B T \sum_{k=1}^{3N-6} \text{tr} \left(\lambda_k^{-1} u_{i,k} u_{i,k}^T \right), \quad (i=1,2,\dots,N) \end{aligned} \quad (5)$$

where λ_k is the k th nonzero eigenvalue, u_{ik} is the amplitude of corresponding eigenvector u_k of the Hessian matrix on residue i . The six lowest zero-valued modes corresponding to rigid body translation and rotation have been omitted. As a result, the B-factors can be obtained by

$$B_i = \left(8\pi^2/3 \right) \langle \Delta \vec{R}_i \cdot \Delta \vec{R}_i \rangle, \quad i=1,2,\dots,N. \quad (6)$$

There are two adjustable parameters in the ANM model, the force constant γ and the cutoff distance r_c . The absolute value of γ has no influence on the relative magnitudes of residue fluctuations: the fluctuation magnitudes are uniformly proportional to $1/\gamma$ as one can see from Eq. (5). Increasing the cutoff distance r_c will give rise to more neighbors for a central residue, leading to a lower and smoother curve for B-factors. We have adjusted these two parameters by fitting to the experimental B-factors of two globular proteins 1vxg sperm whale myoglobin (1.7 Å) and 1pda porphobilinogen deaminase (1.76 Å). A force constant of 0.3 kcal/(mol ·

$\text{\AA}^2$) with an interaction cutoff distance of $r_c = 13.0 \text{ \AA}$ are adopted in the calculations of vibration modes and B-factors. The results are in excellent agreement with experimental data of these two proteins as one can see from Figure 2 (a, b). Because these parameters are derived from globular proteins, there are questions concerning their applications to membrane protein systems. We have addressed these concerns by calculating the B-factors for two membrane protein crystals where high-resolution data are available: 1c3w bacteriorhodopsin ($1.55 \text{ \AA}$) and 1u7g ammonia channel AmtB ($1.4 \text{ \AA}$). The results are shown in Figure 2 (c, d), respectively. It is clear that the B-factor pattern is reproduced excellently by the same parameters. The good agreement for these two cases supports our premise that crystalline disorder is the dominant contribution to the disagreement between computed and measured B-factors for moderate-resolution protein structures. The slight overall lower shift in the calculated B-factors might be caused by residual disorder still present in the crystals or because fluctuations are intrinsically slightly bigger in the membrane environment.

It should be noted that many protein structures from the PDB contain missing residues in the middle of the sequence. These missing residues create breaks in Ca chains of the protein structures, introducing artificially high B-factors for the residues close to the breaks. To reduce such artifacts, the protein structures from PDBs need to be modified by adding the missing Ca residues using molecular dynamics simulations in which the coordinates of the additional residues are optimized to fill the broken gaps whereas the coordinates of the residues from the PDB are kept intact. The interactions among the C α s in the molecular dynamics simulations are the same as those we previously used in simulations in a single-bead protein model.²² These interactions describe reasonably well the backbone configurations of a protein.

RESULTS AND DISCUSSION

The anisotropic network model¹⁶ is applied to the trimeric AcrB transporter X-ray structure described in the PDB entry 1OY6. Figure 3 illustrates the calculated B-factors of one subunit of AcrB using the anisotropic network model as described in the previous section. AcrB is a homotrimer, and the calculated results of the three subunits are identical to one another. Regions with high B-factors (higher than $40 \text{ \AA}^2$) are labeled with numbers 1–11. Their spatial locations are demonstrated in Figure 4. It is interesting to note that region 1 (in Fig. 3), with B-factors $>50 \text{ \AA}^2$, corresponds to the multidrug binding cavity (shown in dark blue in Fig. 4) located at the TM region of AcrB.³ Regions 7, 8, and 10, with B-factors between 45 and $60 \text{ \AA}^2$, correspond to the periplasmic depression area that has been proposed to form an interaction site for the periplasmic linker protein AcrA.⁷ This area has also been identified as a periplasmic multidrug binding site observed in more recent experiments.²³ All these regions are clearly selected by the B-factor calculations based on the elastic network model even though the X-ray structure of AcrB was determined in the absence of bound ligands. The above results lead us to believe that the calculated B-factors may reflect the real dynamical fluctuations in biological systems. Other regions with large motions are located at the top (regions 3 and 9) and bottom ends of the protein (regions 4, 5, and 11). In the TM domains, helix $\alpha 7$ (region 6) is also selected with average B-factor $>50 \text{ \AA}^2$.

The X-ray structure indicates that the AcrB pump consists of three identical subunits, and the three subunits form a jellyfish-like structure. The trimeric AcrB efflux pump possesses a threefold symmetry with threefold axis aligned along the z -direction in our calculation. Each subunit of AcrB consists of 1049 residues. To get a clear picture about the dynamics of this protein, we decompose each B-factor into three components based on the cylindrical coordinate system: z -component along the threefold axis, ρ -component perpendicular to the threefold axis and describes the radial motion of the protein, and θ -component for rotation around the threefold axis. Specifically, the B-factors are decomposed into ρ , θ , and z three components by the following equations:

$$\begin{aligned}
 B_{i\rho} &= \left(8\pi^2/3\right) k_B T \sum_{k=1}^{3N-6} \lambda_k^{-1} \left(u_{ik}^x \cos\theta_i + u_{ik}^y \sin\theta_i\right)^2 \\
 B_{i\theta} &= \left(8\pi^2/3\right) k_B T \sum_{k=1}^{3N-6} \lambda_k^{-1} \left(u_{ik}^{-1} \sin\theta_i + u_{ik}^y \cos\theta_i\right)^2 \\
 B_{iz} &= \left(8\pi^2/3\right) k_B T \sum_{k=1}^{3N-6} \lambda_k^{-1} u_{ik}^z u_{ik}^z
 \end{aligned} \tag{7}$$

where i is the residue index, θ_i is the angle of the position of residue i with respect to x axis.

u_{ik}^x , u_{ij}^y , and u_{ik}^z are x, y, z components of the eigenvector u_{ik} .

The results of the decomposed B-factors are plotted in Figure 5. The results show that most of the motions in AcrB are perpendicular to the threefold axis (i.e., ρ - and θ -components), except in region 2 where it is dominated by motions along the z-direction. Region 5 also has a large z-component along with some ρ - and θ -components. There are 20 missing residues in this region in the original PDB file. These missing residues have been mended by using molecular dynamics simulation. The central multidrug binding cavity (region 1) and the top of the funnel region of AcrB (regions 3 and 9) are found to be primarily breathing motions (ρ -component). However, the periplasmic depression area (regions 7, 8, and 10) is mostly θ movements. In the TM segments (region 6), the motions are found to be mostly rotational and radial motions around the threefold axis.

Although each subunit of AcrB in the homotrimer has > 3,000 modes of vibration, the essential motions in the system are determined by a small fraction of low-frequency modes. This can be seen from Figure 6 in which the simulated B-factors are calculated with the lowest 94 modes of vibration. The slowest modes are expected to have a dominant role in the overall dynamics of the protein. The variation of the B-factors in Figure 6 is very similar to that of total B-factors as shown in Figure 3. However, the background in Figure 6 is much lower than that of Figure 3 indicating that the lowest 94 modes give much better signal-to-noise ratio. Therefore, focusing on the lower frequency modes only can give clearer pictures about the important motions of the protein.

To understand which vibrational modes correspond to particular motions at different regions of the protein, we analyzed the B-factors for different frequency windows. Each window comprises 20 modes in the low-frequency region (below 120 modes). The result is illustrated in Figure 7. The figure shows that the rotational motion of the bottom end of the protein (regions 4, 5, 6, and 11) are mostly from the lowest 14 modes [Fig. 7(a)] whereas the motion of region 11 comes also from higher frequency modes between modes 81 and 100 [Fig. 7(e)]. The motions at the periplasmic depression area (regions 7, 8, and 10) are found to be strongly rotational. The vibrations are mostly localized among modes 21–40 [Fig. 7(b)]. The large motion in region 3 is originating from the frequency window between modes 21–60. The radial motion of region 9 comes mostly from relatively high frequency 41–60 [Fig. 7(c)]. However, the breathing motion of the central binding cavity (region 1) is located at relatively higher frequencies [among the modes 61–120; see Fig. 7(d–f)].

CONCLUSION

Based on the B-factor analysis, we identified important regions for drug binding and transport in AcrB. Region 3, which is located at the top edge of the periplasmic funnel, is found to have enormously large B-factors. The dominant movements at this region are radial and rotational motions. It has been proposed that the top edge of the periplasmic funnel in AcrB interacts with the bottom edge of the periplasmic tunnel of the outer membrane channel TolC during the drug extrusion process.⁷ The extremely large B-factors at the funnel region of AcrB may

indicate that this funnel forms an interaction site, in particular for the TolC channel. If this is the case, this area will be stabilized when AcrB is in complex with TolC.

Regions 7, 8, and 10 highlight four α -helices that form the prominent periplasmic depression in each subunit of AcrB. This depression has been thought to interact with the periplasmic membrane fusion protein AcrA.⁷ The types of movement identified in this area are mostly rotational motions that open and close the periplasmic depression in each subunit of AcrB. It is likely that these modes mimic the motions that AcrB uses to interact with AcrA during drug extrusion. These large B-factors in the AcrB pump also allow us to identify important residues that interact with the AcrA linker protein.

Drug molecules that bind to the central cavity are probably pushed out through the periplasmic domains of AcrB, bypassing periplasm, into the α -helical tunnel of TolC, and finally into the medium. The most direct route for drug extrusion would be through the central pore, which is formed by the pore helix in the periplasmic domain of AcrB, residues 102–115. However, structural investigations of both unliganded and liganded AcrB reveal that these residues make a direct coiled-coil interaction right at the center among the three subunits, and the pore is thus closed.^{3,7} No channel is apparently formed in this region. To understand the role that the central pore has in the drug extrusion process, we study its dynamics by analyzing B-factors of the pore residues. The result shows that the average B-factor of these residues is $<20 \text{ \AA}^2$ for the lowest 100 vibrational modes, indicating the stability of the pore helical structure. In the central binding cavity, the B-factor analysis shows that the motion is dominated by breathing vibrational modes. These radial motions are thought to contribute to the processes of drug binding and transport. In the TM domain, TM7 is found to possess large B-factors. The major movement of this TM helix is mainly rotational and radial motions. Similar movement is also found at the bottom part of the TM domain. Further calculations will be performed to understand the dynamics and function of this region.

In summary, we calculated the dynamics of the trimeric AcrB multidrug transporter using the elastic network model. The calculation is proven to be useful for B-factor analyses in membrane proteins. This technique will be applied to correcting B-factors of other membrane proteins of known structures. It is expected that the technique will allow us to identify important regions, and understand their functions in many membrane proteins. The software used in this study is available from the authors upon request.

ACKNOWLEDGMENTS

Ames Laboratory is operated for the U.S. Department of Energy by Iowa State University under contract W-7405-Eng-82. This work was supported by the Director for Energy Research, Office of Basic Energy Sciences including a grant of computer time at the National Energy Research Supercomputing Center (NERSC) in Berkeley. The work was also supported by a National Institutes of Health Grant R01 GM074027 (to E.W.Y.).

Grant sponsor: Director for Energy Research, Office of Basic Energy Sciences; Grant sponsor: National Institutes of Health; Grant number: R01 GM074027.

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Fig. 1. Structure of the trimeric AcrB transporter with three bound R6G molecules. The figure shows the TM domain (inner and outer leaflets), the periplasmic domain, and the location of cavity, vestibule, pore, and funnel. The bound R6G ligands are located in the central cavity.

Fig. 2.

B-factors of proteins 1vxg (a), 1pda (b), 1c3w (c), and 1u7g (d) calculated by ANM model. The missing residues 1, 2, 49–57 in 1pda, 157–161 in 1c3w, and 13, 14, 23, 44, 82, 92, 146, 200, 301, 328, and 348 in 1u7g are mended by molecular dynamics simulations.

Fig. 3.

B-factors of membrane protein AcrB from experiment (points) and theoretical (ANM) calculations. The large peaks are labeled 1–11 corresponding to the regions shown in Figure 4. The missing residues 1–6, 499–512 are mended by molecular dynamics simulations. Note that we have not scaled the computed values because of the static disorder in the structure.

Fig. 4.

Top, side, and bottom views of the AcrB protein showing regions with large B-factors predicted by ANM calculations. Different colors corresponding to different peaks in the B-factor plot of Figure 3: 1, dark blue; 2, blue; 3, cyan; 4, turquoise; 5, green1; 6, green2; 7, light green; 8, greenish yellow; 9, yellow; 10, gold; 11, red.

Fig. 5. B-factor ρ -, θ -, z -decompositions of membrane protein AcrB. The large peaks labeled 1–11 correspond to the regions shown in Figure 4.

Fig. 6. B-factor of membrane protein AcrB for the lowest 94 vibrational modes. The large peaks labeled 1–11 correspond to the regions shown in Figure 4.

Fig. 7. B-factors of membrane protein AcrB for different frequency windows: **(a)** 7–20, **(b)** 21–40, **(c)** 41–60, **(d)** 61–80, **(e)** 81–100, and **(f)** 101–120. The large peaks labeled 1–11 correspond to the regions shown in Figure 4.