Analysis of the AcrB binding domain of the membrane fusion protein AcrA

Introduction

Antibiotic resistance in bacteria is due in large part to multidrug efflux complexes like the TolC-AcrAB complex in *Escherichia coli*. The crystal structures of two of the three components of this complex have been determined using electron and x-ray diffraction studies, and these structures are believed to be physiologically relevant. TolC and AcrB have been extensively studied and are well characterized. The main impediment to a full understanding of the structure of the entire complex as a unit is the lack of reliable structural information about AcrA or even complete structural information about its homologues. Ideally researchers would like to get a well ordered 3-D crystal of AcrA for use in x-ray diffraction studies, but this goal continues to be elusive. Although a crystal structure would be most beneficial, there are many intermediate steps that will provide useful information about the structure and functions of AcrA.

The TolC-AcrAB complex forms a continuous channel across both the inner and outer membranes when assembled and open. This provides a bridge across the periplasmic space so that compounds harmful to the cell, such as antibiotics, can be transported all the way out of the cell in one coordinated step (8). The range of substrates for AcrB is very broad ranging from bile acids and ethidium bromide to a wide variety of antibacterial drugs. This complex is known to provide *E. coli* and other bacteria with resistance to several commonly used antibiotics such as tetracycline as well as more specialized drugs like ciprofloxacin. A better understanding of the structures and function of all the components of this complex will be helpful in efforts to prevent or at least slow the process of bacterial drug resistance.

Background

The *E. coli* AcrA protein is a member of the membrane fusion protein (MFP) family. This group of proteins also includes *E. coli* EmrA as well as a number of proteins with similar functions in other organisms (5). The precise function for these proteins is not clear, but most of them act in a multidrug efflux pathway. It has been established previously that proteins within the MFP family have a high enough degree of sequential and functional similarity that they can often substitute for each other in multidrug efflux complexes. This conclusion is the basis for the use of structural data on MexA to infer the basic structure of AcrA. Close relatives of AcrA have also been used for studies involving chimeras of AcrA and YhiU to help distinguish the region of AcrA necessary for binding of AcrB (3).

The crystallization process has been quite challenging for those attempting to get structural data on AcrA, but they have been able to create a 2-D crystal. The 2-D crystal was subjected to electron diffraction studies and a tentative structure was proposed (2). The structure obtained from that study was quite low-resolution at about 20Å so there was not a significant amount of new information gleaned from the data but it did provide a positive outlook on the crystallization of AcrA. The only way the AcrA will crystallize thus far is on a lipid-layer, techniques using total lipid extract or standard components of the *E. coli* inner membrane are not successful. Other techniques involving chimeras with other MFPs have been proposed as a possible solution to crystallization difficulties but these methods have not been successful yet.

MexA is a close relative of AcrA that has been successfully crystallized and studied, but the structural information for it is still incomplete. MexA is found in *Pseudomonas aeruginosa* where it is a component of a multidrug efflux complex analogous to the TolC-AcrAB pump in *E. coli*. The partial structure of MexA was determined using x-ray diffraction studies, but the C-

terminus and N-terminus were too disordered to provide any useful structural information in those regions (1). Based on the known structure and the sequence length of the regions for which structure is not available, it is believed that AcrA does indeed span the periplasm. There are 28 uncharted residues between the known region and the fatty acid attachment. The combination of the sequence data and the structural data are enough to postulate a position for binding of AcrA to AcrB in relation to the periplasmic membrane leaflets. Researchers are now aiming to modify the MexA protein in such a way that it will form an ordered crystal where more precise structures of the N and C-termini may be determined.

Another interesting development in the study of AcrA is the discovery of drastic structural changes in response to changes in pH (4). This finding would make sense in the context of the changing environment of the periplasm and might suggest a mechanism for the assembly of the TolC-AcrAB complex. This introduces another reason why residues might need to be highly conserved. Residues required for the conformational change would tend not to tolerate mutation and the change may be required for binding to AcrB. The region required for the movement has not yet been determined due to lack of structural data.

The goal of the experiments proposed here is to identify residues of AcrA that are crucial for its binding interaction with AcrB. It is well established that the presence of AcrA is required for all multidrug efflux via the TolC-AcrAB complex. It is also known that AcrA copurifies with AcrB, indicating that there is a direct binding interaction between the two proteins. We will use site-directed mutagenesis studies to identify the residues for which mutations are not tolerated and use that to draw some conclusions about the nature of the binding interaction with AcrB and perhaps those involved with conformational changes.

Proposed Methods

Identification of residues for site directed mutagenesis. There are several studies that have shown the importance of certain residues of AcrA based on sequence conservation and chimeric analysis data. The data from chimera studies shows that residues 250-297 of AcrA are required for interaction with AcrB (3). This will be that starting point for the site-directed mutagenesis study. The combination of the sequence data from AcrA and the structural information for AcrB can be utilized to narrow down the residues that are potentially involved in the binding site or the proposed conformational change. It has been proposed that the interaction takes place between the periplasmic portion of TolC and the coiled coil region of AcrA around residues 100 to 200. It is likely that AcrB is bound by the other end of the protein in the region we will be mutating which is proposed to be in the beta-sheet conformation. Using the sequence data from AcrA we can predict which residues are likely to be involved in the beta-sheet structure and preferentially investigate those residues. Thus the mutated residues will be within the range of residues 250-297 and also part of a region that is predicted to be in the beta-sheet structure.

Introduction of site specific changes. The procedures for introducing site-mutations onto a plasmid for insertion into *E. coli* is well documented and commonly used. The PCR mutagenesis method will be used due to the high number of mutants desired. The easier methods using various commercial products would be too expensive for this particular application. PCR primers will be designed for each desired mutant. For each targeted residue we will select a similar residue and a drastically different residue. If the targeted residue is an isoleucine for example, we might choose to change it to leucine (AUU to CUU) and to phenylalanine (AUU to UUU). For those mutations in which a conserved replacement is deemed to be tolerated but a

large change is not, we will choose additional similar residues to determine more specifically what types of changes are tolerated at that position and what types of changes are not. We will start with plasmids encoding the coding region of the *acrA* gene with a hexahistidine sequence at the end, in frame with the *acrA* gene, as specified by Zgurskaya et al(7). After introduction of site-specific changes into plasmids, these will be inserted into *E. coli* and grown.

Purification of mutated proteins. Once the mutated proteins have been expressed using plasmids inserted into *E. coli* they will need to be isolated and purified for analysis. This has been done in many recent investigations and I will use the published methods from Zgurskaya et al (7). Their methods for purification of the AcrA-6His protein involve centrifugation and sonication to lyse cells followed by running the lysate over a His-Bind metal chelation resin column. They removed the imidazole using dialysis and ran a sample of the protein on an SDS-PAGE gel to positively identify it as AcrA. A sample of each mutant protein should also be run on a native gel in order to ensure that the protein has been folded correctly. This will not be an absolute measure of correct folding but it will provide a useful control against proteins which do not fold at all or fold incorrectly in such a way that their mobility on the gel is altered in comparison to AcrA-6His

Evaluation of ability of mutated AcrA proteins to bind AcrB. In order to test the ability of the mutated AcrA protein to form a functional complex with AcrB we will test the antibiotic resistance of the *E. coli* strains carrying the mutant proteins as described in the literature (3). We will use the same eight drugs used in the previous studies which include a broad range including dyes like ethidium bromide and antibiotics like tetracycline. We will follow their methods using the *E. coli* strains we prepared by the site-directed mutagenesis procedure. We will begin by testing all of the mutants that have drastic changes at the targeted site. If the drastic change is

tolerated we will not test further. If the drastic change is not tolerated we will test a conserved change strain. If the conserved change is also not tolerated there will be no further tests. If the conserved change is tolerated we will create additional mutants with other similar changes. Mutants will be created and tested until it can be determined what properties are required at that position for a fully functional AcrA.

The final product of this research will be a set of data that describe the tolerated changes at positions within the range of residues 250-297 of the *E. coli* MFP protein AcrA. This data may then be interpreted to produce a model of the residues involved in the interaction of AcrA with AcrB for the purposes of multidrug efflux. Residues that tolerate no change at all are likely to be directly involved in the binding interaction, and residues for which only minor changes are tolerated are most likely to play a supporting role in the binding interaction or a structural role. More research will be necessary to determine the exact positioning of AcrA and AcrB in their contacts with each other, but the experiments outlined here will help in understanding the basic requirements for binding and will be very useful in evaluating crystal structures when they are eventually produced.

References

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