Structural mechanism for multidrug efflux systems

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Structural mechanism for multidrug efflux systems

by

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Abstract

Bacteria such as *Escherichia coli*, *Campylobacter jejuni* and *Neisseria gonorrhoeae* have developed various mechanisms to overcome toxic environments that are otherwise unfavorable for their survival. One important strategy that bacteria use to expel toxic compounds, including heavy metal ions, is the expression of membrane efflux transporters that recognize and actively export these toxic compounds out of bacterial cells, thereby allowing them to survive in extremely toxic conditions. Many of these transporters are multiple drug binding proteins which extrude different toxic chemicals and mediate a phenomenon of multidrug resistance (MDR) in bacteria. The expression of these efflux transporters is tightly controlled at the transcriptional level by transcriptional regulators. A number of these transcriptional regulators are also multidrug binding proteins, which recognize and respond to the same set of toxic chemicals that are expelled by the efflux transporters they regulate. The goal of this Dissertation is to elucidate the structures and fundamental mechanisms that give rise to multiple drug recognition in these efflux transporters and their regulators. We have determined several x-ray structures of these important proteins, including the *E. coli* AcrB efflux transporter, *C. jejuni* CmeR transcriptional regulator and *E. coli* CusB heavy-metal efflux protein. We also crystallized the *N. gonorrhoeae* NorM multidrug transporter and collected the x-ray diffraction data of the crystals. To gain further insight into the mechanism of multiple drug recognition, we examined the binding affinities of AcrB and AcrR to different drugs using fluorescence polarization assays. In this thesis, we will summarize the new findings with AcrB, AcrR, CmeR and CusB, and discuss the structure and function of these efflux transporters and regulators.
CHAPTER 1. General Introduction

Bacterial infections are commonly treated with various classes of antibiotics. The clinical treatment is necessary for curing infectious diseases, but an unintended consequence of the treatment is the selection of bacterial pathogens with elevated levels of resistance to antibiotics. Constant emergence and spread of antibiotic resistance has become a major threat to the health of humans and animals (1). Bacterial organisms utilize multiple mechanisms to combat antibiotics and antimicrobial agents. One important mechanism that gives rise to multidrug resistance (MDR) is the expression of multidrug efflux transporters that are capable of reducing the intracellular concentration of toxic compounds (2-6), thereby allowing the bacterial cells to survive in unfavorable environments. Often a single organism can harbor multiple multidrug efflux transporters to secure its survival. Currently, these multidrug efflux transporters can be divided into five different families: the resistance-nodulation-division (RND) family (7), the ATP-binding cassette (ABC) superfamily (8), the small multidrug resistance (SMR) family (9), the major facilitator (MF) superfamily (10-12), and the multidrug and toxic compound extrusion (MATE) family (13-14). In Gram-negative bacteria, the most relevant multidrug efflux systems in terms of resistance to clinically important agents are members of the RND family (15), although members of the MFS, SMR, and MATE families also show a limited ability to promote resistance to some antibiotics (16).

Typically, an RND transporter works in conjunction with a periplasmic membrane fusion protein (17) and an outer membrane channel (18). The resulting tripartite efflux complex spans the inner and outer membranes of a bacterium and exports substrates directly out of the bacterial cell. This process is driven by proton import, which is catalyzed by the inner membrane RND transporter. As a Gram-negative bacterium, *E. coli* possesses seven transporters that belong to the RND family. These transporters are responsible for the intrinsic tolerance of antibiotics and toxic compounds (7). Among these seven transporters, six of them, AcrB (19-23), AcrD (24), AcrF (25), MdtB (26, 27), MdtC (26, 27) and YhiV (28), are multidrug efflux pumps. They belong to the hydrophobic and amphiphilic efflux RND (HAE-RND) protein family (7). *E. coli* consists of only one heavy-metal efflux RND (HME-RND)
transporter, CusA, which specifically recognizes and confers resistance to Ag$^+$ and Cu$^+$ ions (29, 30).

The novel MATE family of transporters was identified only quite recently. The first well-characterized MATE transporter is *Vibrio parahaemolyticus* NorM, a multidrug Na$^+$-antiporter that confers resistance to dyes, fluoroquinolones and aminosugarides (14). Recently, NorM of *N. gonorrhoeae* and *N. meningitidis* have been found and characterized (31, 32). Deletions of these pumps resulted in an increased susceptibility to cationic and neutral drugs (31). Another MATE transporter, YdhE from *E. coli* has also been demonstrated to confer resistance to cationic and neutral antimicrobials (32, 33). Currently, little is known about the structure and regulation of the MATE family. Thus, there is a strong rationale for the investigation of this transporter family.

The expression of multidrug efflux transporters is tightly controlled at the transcriptional level by regulators (2). Many of these transcriptional factors are multidrug binding proteins, which recognize and respond to the same set of toxic chemicals that are exported by the transporters they regulate (34). These transcriptional factors act as cytosolic chemical sensors and respond to threatening levels of toxic compounds (35, 36). In bacteria, transcriptional regulation involves either one-component or two-component regulatory systems. Two-component regulatory systems control protein expression through the function of a membrane-bound sensor kinase and a cytoplasmic response regulator, which is a DNA-binding protein (36-38). The membrane-bound kinase is responsible for receiving external signals and transmitting the information into the cell by phosphorylating the DNA-binding protein. The phosphorylated DNA-binding protein then modulates gene transcription by interacting with its cognate DNA. A key feature of two-component regulatory systems is the phosphorylation between sensor kinase and response regulator. One-component bacterial transcriptional regulators modulate gene expression levels using a single two-domain protein where one domain receives signals and the other domain binds specific DNA sequences to regulate transcription (36). Information flow between the two domains is through conformational changes, contrasting the phosphorylation events required in two-component systems. Structural analyses revealed that almost 95% of all known prokaryotic transcriptional factors employ the helix-turn-helix (HTH) motif to bind their target DNAs (36). Prokaryotic
transcriptional regulators are classified in families based on their functional and sequence similarities. One such family is the TetR family of transcriptional regulators (36). Members of the TetR family are two-domain proteins which possess an N-terminal HTH DNA-binding motif and a C-terminal ligand regulatory domain. Many of these regulators control the expression of MDR efflux transporters that are required for bacteria to adapt to environmental stresses. These transporters protect bacterial cells from deleterious compounds by actively extruding these compounds as they enter the cells.

Understanding the action mechanisms of these efflux transporters and their transcriptional regulators is vital due to the potential that these proteins can offer for new drug targets. Of all known multidrug efflux transporters, the E. coli AcrB multidrug efflux pump shows the widest substrate specificity, ranging from most of the currently used antibiotics, disinfectants, dyes, detergents, to simple solvents (39, 40). Thus, AcrB offers one of the best systems to understand the broad chemical specificity of these transporters. In Chapter 2 of this dissertation, we describe the crystal structures of four AcrB mutants. These mutant proteins have allowed us to depict one of the transient conformational states of the transporter during the transport cycle.

Chapter 3 concerns the drug-transporter interaction in the AcrB efflux transporter. Our experimental results indicate that this transporter binds antimicrobials with dissociation constants in the micromolar region. Remarkably, we observed that the AcrR transcriptional regulator, which modulates the expression of AcrB in E. coli, interacts with the same set of toxic chemical compounds as AcrB with strikingly similar affinities. Chapter 4 demonstrates our study on how this regulator interacts with different antimicrobial agents.

In Chapter 5 we switch our gear to elucidate the crystal structure of the Campylobacter jejuni CmeR transcriptional regulator, which controls the expression of the tripartite CmeABC RND-type multidrug resistant efflux transporter. Our findings reveal novel structural features of this regulator and provide new insight into the mechanisms of ligand binding and CmeR regulation.

Chapter 6 concerns the crystallization and preliminary x-ray diffraction analysis of the multidrug efflux transporter NorM from the pathogen N. gonorrhoeae. NorM belongs to the most recently classified MATE family of transporters and recognizes a number of cationic and
neutral antimicrobial agents. The crystallographic data potentially will allow us to produce the first structural model of this new transporter family.

Chapter 7 describes the crystal structure of the CusB membrane fusion protein which associates with the inner membrane efflux transporter CusA and an outer membrane channel CusC to form the tripartite CusABC efflux complex in *E. coli*. This RND-type efflux system specifically recognizes and confers resistance to Cu(I) and Ag(I) ions.

References


CHAPTER 2. Conformation of the AcrB Multidrug Efflux Pump in Mutants of the Putative Proton Relay Pathway


Chih-Chia Su, Ming Li, Ruoyu Gu, Yumiko Takatsuka, Gerry McDermott, Hiroshi Nikaido, and Edward W. Yu

ABSTRACT

We previously reported the X-ray structures of wild-type *Escherichia coli* AcrB, a proton motive force-dependent multidrug efflux pump, and its N109A mutant. These structures presumably reflect the resting state of AcrB, which can bind drugs. After ligand binding, a proton may bind to an acidic residue(s) in the transmembrane domain, i.e., Asp407 or Asp408, within the putative network of electrostatically interacting residues, which also include Lys940 and Thr978, and this may initiate a series of conformational changes that result in drug expulsion. Herein we report the X-ray structures of four AcrB mutants, the D407A, D408A, K940A, and T978A mutants, in which the structure of this tight electrostatic network is expected to become disrupted. These mutant proteins revealed remarkably similar conformations, which show striking differences from the previously known conformations of the wild-type protein. For example, the loop containing Phe386 and Phe388, which play a major role in the initial binding of substrates in the central cavity, becomes prominently extended into the center of the cavity, such that binding of large substrate molecules may become difficult. We believe that this new conformation may mimic, at least partially, one of the transient conformations of the transporter during the transport cycle.
INTRODUCTION

The *Escherichia coli* AcrB multidrug efflux pump (10, 11) is a member of the resistance-nodulation-division transporter family (18). It recognizes many structurally unrelated toxic compounds and actively engages to extrude them from cells. Its crystallographic structure was solved by Murakami et al. (13) in 2002. We previously reported the X-ray structures of AcrB in the presence of four different ligands (21, 22). The structures showed that these ligands bind to the wall of the extremely large central cavity in the transmembrane region of the pump. This binding presumably corresponds to the first step in the drug extrusion process, since drug molecules then have to pass through the periplasmic domain of AcrB and eventually reach the outer membrane channel TolC. A subsequent study of the efflux pump by crystallization of a mutant AcrB protein with an N109A mutation with five structurally diverse ligands (20) indicated that AcrB contains at least two distinct binding sites. These five ligands not only bind to various positions of the central cavity but also bind to residues lining the deep external depression formed by the C-terminal periplasmic domain.

AcrB is a proton motive force-dependent multidrug efflux pump that functions via a drug/proton antiport mechanism (23). Coupled with the outward movement of drug molecules, protons have to flow inward (towards the cytoplasm) to energize the efflux process. AcrB contains two acidic residues, Asp407 and Asp408, in the transmembrane (TM) helix TM4 and one basic residue, Lys940, in TM10, and these three residues appear to constitute a salt-bridged (and/or hydrogen-bonded) network (13, 22) (Fig. 1). The presence of such residues often means that they play an important functional role, presumably in the translocation of protons.

For MexB (a homolog of AcrB) of *Pseudomonas aeruginosa*, it has been shown that Asp407, Asp408, and Lys939 (corresponding to Asp407, Asp408, and Lys940 of AcrB) are indeed essential for transport function (7), and this Asp-Lys-Asp triad was also found to be essential in *E. coli* AcrB (14). Recently, we found that Thr978 of AcrB TM11, located close to the triad, is also essential for function (17); this residue may also be a component of the putative network of tightly interacting residues just mentioned (Fig. 1).
During the translocation of the ligand, active transporters must go through significant conformational changes, which are coupled to the expenditure of energy. With transporters that use ATP hydrolysis as an energy source, one can attempt to trap the transporter in one of the transient conformations by using vanadate-ADP (5, 16). However, similar approaches are not feasible with transporters such as AcrB, which is energized by proton motive force. We reasoned that proton translocation may perturb the salt bridge/H-bonding interactions within the D407-K940-T978-D408 complex and that this transient state of AcrB might be mimicked by replacing one of these residues with alanine, which cannot be protonated or deprotonated. We report here that the D407A, D408A, K940A, and T978A mutations cause remarkably similar and extensive alterations in the conformation of AcrB.

**MATERIALS AND METHODS**

**Construction of D407A, D408A, K940A, and T978A mutants.** Mutations were introduced by the method described in the accompanying paper (17). All of the mutant acrB genes contained a sequence coding for four additional histidine residues at the C terminus, and each of the proteins therefore had a hexahistidine sequence at the end (together with two histidine residues supplied by the native AcrB protein).

**Purification of AcrB.** The mutant AcrB proteins were overproduced in *E. coli* BL21-Gold(DE3) cells (Stratagene), using a plasmid derived from pSPORT1 (Invitrogen). Cells were grown in 6 liters of LB medium with 100 µg/ml ampicillin. Cells were disrupted with a French pressure cell. The membrane fraction was collected and washed twice with high-salt buffer containing 20 mM sodium phosphate (pH 7.2), 2 M KCl, 10% glycerol, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride and once with 20 mM HEPES-NaOH buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride. The membrane proteins were then solubilized in 1% n-dodecyl-β-D-maltoside (wt/vol). Insoluble material was removed by ultracentrifugation at 370,000 x g. The extracted protein was purified with hydroxyapatite and Cu²⁺-affinity columns (23).
Crystallography. Crystals of the D407A, D408A, K940A, and T978A mutants were grown by hanging-drop vapor diffusion at 25°C. A protein solution containing 30 mg/ml mutant protein in 20 mM Tris (pH 7.5), 0.1% n-dodecyl-β-D-maltoside, and 20 mM dithiothreitol was mixed with an equal volume of a reservoir solution containing 6.5 to 8.5% polyethylene glycol 3000, 8% glycerol, and 30 mM sodium citrate (pH 5.6) or 30 mM potassium citrate (pH 6.5). Crystals appeared in the drops within 4 days. Cryoprotection was achieved by raising the glycerol concentration stepwise to 35%, with a 5% increment at each step. The conditions used for crystallization were essentially identical to those used earlier for the wild-type AcrB protein (22), except for the pH of the Tris buffer used. When the wild-type protein was crystallized under the conditions described here, a structure identical to that reported earlier was obtained (unpublished results).

All X-ray intensity data sets were collected at the Advanced Light Source at Lawrence Berkeley National Laboratory (beamline 8.2.1) at a cryogenic temperature (100 K). The diffraction data were processed with DENZO and scaled with SCALExPACK (15). The crystals of the AcrB mutants took the R32 space group, with the unit cell dimensions listed in Table 1. Initially, the overall structures of the AcrB mutants were determined by molecular replacement, using the MolRep program (19) in the CCP4 package. The wild-type AcrB structure (Protein Data Bank no. 1OY6) was used as a search model. Before refinement, 5% of all data were set aside for cross-validation (2). The model refinements were performed using CNS (3) and CCP4 (4). Model rebuilding was conducted using the program O (8).

Labeling of purified CL-F386C AcrB<sup>His</sup> mutant proteins with MIANS and fluorescence measurements. The acrB gene was first modified by converting the codons for two intrinsic cysteines (Cys493 and Cys887) to those for serines by site-directed mutagenesis, producing cysteine-less (CL) AcrB; this mutant protein appeared to be fully functional in providing drug resistance, as reported earlier for proteins with alanine mutations of the same residues (6). Phe386 in CL AcrB was then converted to cysteine, and another mutation in the proton relay region was introduced, when necessary, in the same way. The purified CL-F386C AcrB<sup>His</sup> proteins (3 to 4 mg/ml in 20 mM HEPES-KOH buffer containing 50 mM NaCl, 0.02% n-dodecyl-β-D-maltoside, and 10% glycerol [pH 7.5]) were diluted in the assay buffer (20 mM
HEPES-KOH [pH 7.5] containing 50 mM NaCl and 0.02% \( n \)-dodecyl-\( \beta \)-d-maltoside) at a concentration of 0.5 \( \mu \)M (57 \( \mu \)g/ml). The reaction was initiated by the addition of 2-(4'-maleimidylanilino)naphthalene 6-sulfonic acid sodium salt (MIANS; Molecular Probes) to a final concentration of 5 \( \mu \)M, and fluorescence was monitored continuously at room temperature with an RF-5301PC spectrofluorophotometer (Shimadzu) at an emission wavelength of 430 nm (excitation, 330 nm). A similar labeling experiment was also carried out by using 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM; Molecular Probes).

**Protein structure accession numbers.** The coordinates for the protein structures have been deposited in the Protein Data Bank (PDB) under accession numbers 2HQC (D407A mutant), 2HQD (D408A mutant), 2HQF (K940A mutant), and 2HQG (T978A mutant).

**RESULTS**

**Crystallographic structures of mutants of the putative proton relay system.** We solved the three-dimensional structures of the AcrB D407A, D408A, K940A, and T978A mutants by X-ray crystallography through molecular replacement. The structures were determined with a maximum resolution of 3.38 to 3.65 Å. The overall \( R_{\text{work}} \) values were between 25.1 and 27.1\%, and \( R_{\text{free}} \) values were between 27.5 and 30.3\%. Refinement statistics are shown in Table 1. The overall structures of these mutant proteins were very similar to each other. Each mutant protein formed a homotrimer, with its threefold symmetry axis passing through the center of the trimer. Compared with wild-type AcrB, the mutant transporters showed some unique conformational changes, as described below.

**Short-range alterations.** Electron density omit maps showed that some of the tight interactions among four residues, namely, Asp407, Asp408, Lys940, and Thr978 (Fig. 1), became profoundly disturbed in the mutants. As shown in Fig. 2 (top panel), in the wild-type protein the side chains of some of these residues show clearly interpretable electron densities, even at the modest overall resolution of our structures, presumably because they are immobilized by the strong electrostatic interactions between them. However, in the mutant
proteins (Fig. 2, bottom panel, shows the same region in the D408A mutant protein as an example), much less detail of the side chain density is seen, most likely because the tight interaction network was disturbed.

This disorder in the network region, introduced by the mutations, apparently spreads into the neighboring regions. Thus, in all four mutants, extensive changes occur in the backbone atoms in the part of TM4 that is proximal to residue 407 (Fig. 3). Thus, the regular H-bonding interactions of the backbone NH and CO groups characteristic of the α-helix (Fig. 3, left panel) become distorted or disrupted (the right panel of Fig. 3 shows the D407A mutant as an example; similar changes are also found in the other mutants), as also seen in the disappearance of regular helical structures in the upper part of TM4 in Fig. 4 (bottom panel), where the structure of the TM domain of the K940A mutant is given as an example. The disordering of the proximal part of TM4, as illustrated in Fig. 3, makes this segment longer, also creating a drastic change in the positions of residues of the loop between TM helices 3 and 4 (noted by an arrow on the left in Fig. 5). This loop is located close to the ceiling of the central cavity, and the details, which could be established unequivocally because of the electron density of the phenyl groups in Phe386 and Phe388, are shown in Fig. 6B (panel A shows the electron density maps of the wild-type protein and the K940A mutant protein). Compared with the wild-type structure (Fig. 6B, left panel), the loops of all mutants, formed by residues 384 to 393 between TM3 and TM4, intrude prominently into the center of the cavity (Fig. 6B, right panel). The change includes residues Phe386 and Phe388 coming about 6 to 7 Å closer to the center of the cavity, and thus also to their counterparts from the other subunits of the trimer. For example, the distances between the CZ carbon atoms of the phenyl groups of Phe386 and Phe388’ in the D407A, D408A, K940A, and T978A mutants are 6.7 Å, 6.0 Å, 6.2 Å, and 7.4 Å, respectively, in contrast to 16.2 Å in the wild-type protein. These three phenylalanines essentially decrease the size of the upper part of the central cavity by forming a septum-like structure (Fig. 6C), resulting in an opening of only about 6 Å in diameter at this region, in contrast to the nearly 20-Å-diameter opening in the wild-type protein.
**Long-range alterations.** The local changes were propagated to produce a number of remarkably extensive long-range alterations, as shown in Fig. 4. Thus, looking at the K940A mutant protein, the kinked TM helix 5 becomes disordered such that the helical structure before the kink totally disappears, and even the portion after the kink becomes strongly disordered. The following TM6 helix also becomes shortened. Furthermore, the helix connecting the N-terminal and C-terminal halves of the transmembrane domain, i.e., I3 (13), again becomes drastically shortened. The TM helices of the C-terminal half (TM7 through TM12) do not undergo extensive disordering, except that helix 11 (containing Thr978) becomes somewhat shorter and the C-terminal end of helix 12 becomes disordered. All of these changes occur with remarkable regularity in all four mutants (K940A, D407A, D408A, and T978A). As part of this global conformational alteration, we found that the upper half of TM5 shifts upward about 2 Å, just like the corresponding part of TM4. Significant backbone movement was also found in the flexible loop, i.e., residues 29 to 32, above TM1 (the electron density map of this region is also shown in Fig. 6A, and the shift is highlighted with an arrow on the right in Fig. 5). This loop forms one side of the vestibule through which ligands are hypothesized to enter the central cavity (13, 21, 22). The displacement can be interpreted as a rotation of this portion of the loop, with the rotational axis passing through the C- atoms of residues 28 and 32. The side chain of Leu30, which is at the center of this displaced region, appears to have been flipped about 100° from the central cavity. The rotation also shifts the locations of the C- atoms of Leu30 and Pro31 about 5 Å away from their original positions. The movement of this region occurs in a direction opposite (that is, away from the cavity) from that of the nearby loop between TM3 and TM4 mentioned earlier (into the cavity).

Many regions of the periplasmic domain are also altered. It is intriguing that the loop that forms the bottom and part of the "left" wall of the periplasmic drug binding site (20) is shifted significantly (indicated by an arrowhead in Fig. 5). The side chain of one of the residues that appear to be involved in ligand binding, Glu673 (see Fig. 5 in reference 20), is part of this shifted region, and in our model its side chain is moved away from the position of bound ligand about 6 Å in comparison with the position in the wild-type protein.
Accessibility of the loop between TM3 and TM4. The conformational alterations observed in the mutant AcrB proteins were extensive. Although the crystallization conditions were similar to those employed earlier (21, 22), we wanted noncrystallographic evidence to strengthen our conclusion that the region involving Phe386 and Phe388 is indeed altered strongly in its conformation in the mutant proteins. We therefore mutated the Phe386 residue to cysteine and examined the accessibility of this residue for modification with fluorescent maleimide reagents. As shown in Fig. 7, Cys386 mutant AcrB proteins containing additional mutations in the putative proton relay complex (D407A, D408A, K940A, and T978A) were modified by MIANS significantly more rapidly than the protein not containing any second mutation. Because the fluorophore of MIANS (anilinonaphthalene sulfate) is affected by its environment in terms of its fluorescence, we also used a coumarin maleimide (CPM), which is much less sensitive to its environment, and obtained similar results (not shown). These results suggest that the conformation of the region between TM3 and TM4 is indeed altered in the D407A, D408A, K940A, and T978A mutants.

DISCUSSION

In previously determined structures of AcrB, the side chains of Asp407, Asp408, Lys940, and Thr978 are close to each other and appear to form a salt bridge/hydrogen-bonding network (Fig. 1). It may be postulated that AcrB in this "resting-state" conformation is ready to bind substrates, as the central cavity of this conformer has indeed been shown to bind various drugs (22).

As a working hypothesis, we assumed that the ε-amino group of Lys940 is protonated and the carboxyl groups of Asp407 and Asp408 are deprotonated, in view of their close proximity (17). During the process of proton translocation, one of the carboxyl groups, perhaps that of Asp407, can be postulated to become protonated. This is similar to what has been assumed for many active transporters energized by proton motive force (for example, see the case of the lactose permease LacY [1]). This protonation of an acidic residue is expected to disturb the tight salt bridge/hydrogen-bonding network of the four residues, distort the positions of these residues relative to each other, and initiate a series of
conformational changes that would result in the transport of drugs, as stated in the accompanying paper (17).

We undertook this study on the assumption that disturbing the interactions among Asp407, Asp408, Lys940, and Thr978 might in some way mimic the consequences of protonation of one of the carboxyl residues. Indeed, we found that conversion of any of these residues to alanine produced a widespread conformational alteration (Fig. 3 to 6). It is also remarkable that mutation of any of the four residues produced nearly identical new conformations, suggesting that the trigger for change is the disruption of the same salt bridge/H-bonding network. Although it may be argued that the replacement of a charged residue with an uncharged Ala residue may disturb the protein structure simply because of the unfavorable thermodynamics of insertion of the remaining charged residues into the membrane, without any relation to H+ translocation, the same altered conformation in the T978A protein, where charged residues have not been altered, refutes this interpretation.

The new conformation shows that the TM domains of the three protomers still form a large central cavity with a diameter of about 35 Å. However, the diameter of this central cavity decreases to about 6 Å in a region close to the ceiling due to the protrusion of the loop containing Phe386 and Phe388 (Fig. 6). This is the loop that forms a multidrug binding site in wild-type AcrB (22), but this region may become too tight to bind large drugs in these AcrB mutants. If we assume that the new conformation mimics one of the transient stages after the binding of drugs by resting-state AcrB, it may be that the conformational alteration induced by protonation pushes the drug molecules away from the initial site to a new position closer to the final stage of their expulsion. Drug binding studies, both biochemical and crystallographic, are obviously needed to follow up this hypothesis.

Compared with the trimer of wild-type AcrB, there is an increase in intersubunit distance in the mutant homotrimers. For example, the distance between the top regions of TM1 and TM8’ from adjacent subunits is increased about 2 Å in the K940A structure. This change opens the vestibule located above these TM helices some 3 Å. It also enlarges the bottom of the cavity opened to the cytoplasm by 2 Å. Similar changes were found in the other
mutant AcrB proteins. Anisotropic network model calculations (9) showed that the motion in the central cavity of AcrB is dominated by breathing vibrational modes. We suspect that the expansion of the AcrB mutants mimics this breathing motion.

Our hope was that the conformation of the mutant proteins would give us not only a glimpse of the transitional states of the AcrB transporter during a normal cycle, but also hints on the pathway of drug molecules during export, especially within the periplasmic domain. However, alterations were not extensive in the conformation of the periplasmic domain (Fig. 5), and the reasons for this outcome are not known.

REFERENCES


Figures and Captions

FIG. 1. Putative salt bridge/H-bonding network (D407-D408-K940-T978) in the wild-type AcrB protomer, based on PDB file 1IWG (15). The view is along the line perpendicular to the membrane surface, from the periplasmic side. In spite of the modest resolution of the overall structure, the electron densities of some side chains can be seen clearly (see Fig. 2, top panel). The stick model in this figure as well as that in Fig. 6B was produced by the program PyMol (W. L. Delano, PyMol Graphic System [www.pymol.org]). The locations and sizes of the cross sections of TM4, TM10, and TM11 shown are crude approximations added simply to aid understanding.
### TABLE 1. Data collection and crystallographic analysis of AcrB mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Cell constants (Å)</th>
<th>Resolution (Å)</th>
<th>Completeness (%)</th>
<th>$R_{sym}$ (%)</th>
<th>$R_{work}/R_{free}$ (%)</th>
<th>No. of reflections</th>
<th>1/ (I)</th>
<th>φ</th>
<th>Total Unique</th>
</tr>
</thead>
<tbody>
<tr>
<td>D407A mutant</td>
<td>$a = b = 145.4, c = 514.5$, $α = β = 90, γ = 120$</td>
<td>3.56 (3.78-3.56)</td>
<td>100 (94.7)</td>
<td>10.2</td>
<td>27.1/29.4</td>
<td>1,086,717</td>
<td>41,936</td>
<td>3.9</td>
<td></td>
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<tr>
<td>D408A mutant</td>
<td>$a = b = 145.0, c = 513.7$, $α = β = 90, γ = 120$</td>
<td>3.65 (3.78-3.65)</td>
<td>94.7 (92.3)</td>
<td>10.6</td>
<td>26.1/30.3</td>
<td>618,639</td>
<td>23,658</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>K940A mutant</td>
<td>$a = b = 145.6, c = 519.7$, $α = β = 90, γ = 120$</td>
<td>3.38 (3.56-3.38)</td>
<td>99.9 (99.0)</td>
<td>7.8</td>
<td>25.1/28.0</td>
<td>880,107</td>
<td>43,054</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>T978A mutant</td>
<td>$a = b = 144.9, c = 518.6$, $α = β = 90, γ = 120$</td>
<td>3.38 (3.56-3.38)</td>
<td>99.1 (94.6)</td>
<td>9.3</td>
<td>25.4/27.5</td>
<td>1,112,163</td>
<td>42,367</td>
<td>2.7</td>
<td></td>
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*a* All mutants belong to space group R32. Numbers in parentheses are for the highest-resolution shells.
FIG. 2. Side view of the putative proton relay network involving TM4 and TM10. Electron density omit maps were calculated at 1 Å. (Top) In wild-type AcrB, some of the side chains are visible, presumably because of the strong electrostatic interactions among these residues. (Bottom) In contrast, in mutants (the D408A mutant is shown as an example), much less detail can be seen, presumably because of the weakening and abolition of the electrostatic interactions.
FIG. 3. Structure of the N-terminal portion of TM4. The view is from the side (in a direction parallel to the membrane surface). The hydrogen bonds (green dotted lines) were calculated by the program DeepView, and the picture was drawn by using DeepView and POV-ray. The wild-type structure (left) and the D407A mutant structure (right) were aligned for the best overall fit of the entire protein backbone by DeepView and then horizontally displaced for a better view. In the wild-type AcrB protein (left), the backbone NH of Asp407 is H-bonded to the backbone C=O of Gly403, four residues back, as it should be in the regular α-helical structure. However, in the D407A mutant AcrB protein (right), the corresponding NH of Ala407 appears to H-bond to the C=O of not only Gly403 but also Leu404, distorting the helix. Further upward, the H-bonding interactions involving NH groups of Leu405, Leu404, and Gly403 in the wild-type protein are totally lost in the mutant protein. This distortion of the α-helix extends the chain, such that the top of TM4 (near the top of the figure) becomes higher in position in the mutant protein by nearly 2 Å, as shown.
FIG. 4. Secondary structures in TM domains. The figure was drawn by using the Cn3D program (National Center for Biotechnology Information) after alignment of the three-dimensional structures of the wild type (PDB file 1IWG) and the K940A mutant (this study) through the Vector Alignment Search Tool (12), available at the National Center for Biotechnology Information website. Note that the assignment of the secondary structure was done in a uniform manner by the Vector Alignment Search Tool program, independent of the annotations in the PDB files. The TM helices are numbered in the wild-type structure.
FIG. 5. Global conformation of AcrB protomers, showing the deviation of the K940A structure from the wild-type structure. The structures were aligned for the best global fit, using backbone atoms, by the Iterative Magic Fit feature of the DeepView program, and the mutant structure was colored in rainbow colors (with red showing the most deviation) by using the "color by RMS deviation" feature of the program, whereas the wild-type structure is shown in white. The structure is shown so that the helices on the right side correspond to the N-terminal ones (TM1 through TM6) and those on the left side correspond to the C-terminal ones (TM7 through TM12). The structure is tilted slightly from the membrane perpendicular in order to show the loop between TM3 and TM4 more clearly. The shift of this loop between TM3 and TM4 is indicated by an arrow on the left, and that of the loop of residues 29 to 34 is shown by an arrow on the right. The shift of the loop at the bottom of the periplasmic ligand binding site (23) is indicated by an arrowhead. Red sections at the extreme left portion of the figure are an artifact caused by the absence of some residues in the wild-type structure. The drawing was done by using DeepView and POV-ray.
FIG. 6. Structure of the loop between TM3 and TM4. (A) Simulated annealing composite omit map of the K940A mutant contoured at 1.0 σ. The figure shows the locations of Phe386 and Phe388 in the central cavity and of residues along the left side of the vestibule. (B) Models of the loop (residues 385 to 389) viewed along the threefold axis (perpendicular to the membrane surface) from the periplasmic side. The left panel shows the structure in wild-type AcrB (from PDB file 1IWG) (15), and the right panel shows the structures in mutant AcrB proteins (D407A, green; K940A, mauve; and T978A, orange) overlaid on top of the wild-type structure (yellow). (C) Vertical slab (5 Å thick) of the AcrB trimer, showing the essential closure of the top portion of the central cavity by the loop between TM3 and TM4 in the K940A mutant protein (right). The same slab of the wild-type trimer is shown on the left for comparison. The slice plane goes through the approximate center of the central cavity as well as through the Pro833 residue in protomer A and Thr145 in protomer B. The picture was generated by the Deep View program followed by POV-ray.
FIG. 7. Reaction of purified CL-F386C AcrB<sup>His</sup> mutants with the fluorescence probe MIANS. MIANS labeling was carried out with 0.5 µM purified protein in 2 ml of 20 mM HEPES-KOH (pH 7.5)-50 mM NaCl-0.02% dodecyl maltoside. Reactions were initiated by the addition of MIANS to a final concentration of 5 µM, and the fluorescence increase was recorded continuously at 430 nm (excitation, 330 nm). Mutants were as follows: 1, CL-F386C; 2, CL386-N109A; 3, CL386-D407A; 4, CL386-D408A; 5, CL386-K940A; and 6, CL386-T978A.
CHAPTER 3. Ligand-transporter interaction in the AcrB multidrug efflux pump determined by fluorescence polarization assay


Chih-Chia Su and Edward W. Yu

Abstract

The AcrB of *Escherichia coli* pumps out a wide range of compounds, including most of the currently available antibiotics, and contributes significantly to the serious problem of multidrug resistance of pathogenic bacteria. Quantitative analysis of drug efflux by this pump requires the measurement of the affinity of ligands. Yet there has been no success in determining these values. We introduce here an approach of steady-state fluorescence polarization to study the interactions between four different ligands and the purified AcrB transporter in a detergent environment. Our assays indicate that the transporter binds these drugs with $K_D$ values ranging from 5.5 to 74.1 µM.
1. Introduction

The emergence of multidrug-resistant pathogenic bacteria is a major current problem in public health. In gram-negative bacteria, much of the multidrug resistance phenotype is caused by the increased expression of multidrug efflux pumps. These pumps fall into several families, small multidrug resistance (SMR), major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), and resistance-nodulation-division (RND). The members of the RND superfamily [1] pump out the widest range of compounds, and are usually the major contributor to both the intrinsic antibiotic resistance, and when overexpressed, the increased resistance levels of multidrug resistant strains [2], as was recognized already in 1993 [3] and 1994 [4]. However, quantitative analysis of the function of RND efflux pumps could not even be attempted during the last 14 years, because all attempts to measure the kinetic constants of the pump resulted in failure.

We here use a relatively simple methodology to quantitatively monitor interaction between a membrane protein, which in this study is the AcrB multidrug efflux pump of *Escherichia coli* [5], and its fluorescent ligands in detergent solution. Binding of a fluorescent ligand to a protein causes a decrease in rotational motion due to an increase in the size of the protein–ligand complex as compared to that of the free ligand, and thus results in increases in the polarization of fluorescence of the bound ligand. Fluorescence polarization is defined as

$$ P = \frac{I \parallel - I \perp}{I \parallel + I \perp} $$

where $P$ is the observed polarization, and $I \parallel$ and $I \perp$ represent the intensity of emissions parallel and perpendicular to the incident polarized light.

AcrB [5] is a prototypical member of the RND family of transporters [1]. It recognizes many structurally unrelated compounds, including most of the currently available antibiotics and chemotherapeutic agents, detergents, dyes, and simple solvents, and actively engages to extrude them from cells [6]. This inner membrane efflux pump interacts with a
periplasmic membrane fusion protein, AcrA [7], and an outer membrane channel, TolC [8], to mediate the extrusion of toxic compounds directly into the external medium, across both membranes of *E. coli*.

The structure of AcrB has been studied extensively [9], [10], [11], [12], [13], [14], [15] and [16]. Using the purified AcrB multidrug efflux pump, we succeeded for the first time in determining the affinity of ligands for the pump, and further in demonstrating the competition between ligands for the binding process. This study will be the first step for the quantitative analysis of the kinetics of the most important class of multidrug efflux pumps in gram-negative bacteria.

2. Materials and methods

2.1. Purification of AcrB

The AcrB protein that contains a 4×His tag at the C-terminus was overproduced in *E. coli* BL21-Gold (DE3) cells (Stratagene) using the plasmid derived from pSPORT1 (Invitrogen) [17]. Cells were grown in 6 L of LB medium with 100 µg/ml ampicillin. Cells were disrupted with a French pressure cell. The membrane fraction was collected and washed twice with high salt buffer containing 20 mM sodium phosphate (pH 7.2), 2 M KCl, 10% glycerol, 1 mM EDTA and 1 mM phenylmethanesulfonyl fluoride (PMSF), and once with 20 mM HEPES–NaOH buffer (pH 7.5) containing 1 mM PMSF. The membrane proteins were then solubilized in 1% (w/v) *n*-dodecyl-β-d-maltoside (DDM). Insoluble material was removed by ultracentrifugation at 370 000 × *g*. The extracted protein was purified with hydroxyapatite, Cu²⁺-affinity and G-200 sizing columns [10] and [18].

2.2. Fluorescence polarization assays

Fluorescence polarization assays [19] and [20] were used to determine the drug binding affinities of AcrB. The experiments were done using a ligand binding solution containing 20 mM Tris (pH 7.5), 0.05% DDM, and 1 µM ligand [rhodamine 6G (R6G), ethidium (Et), proflavin (Pf), or ciprofloxacin (Cip)]. The AcrB protein solution in 20 mM Tris (pH 7.5),
0.05% DDM, and 1 µM ligand was titrated into the ligand binding solution until the polarization (P) became unchanged. In this assay, the protein–drug interaction would reach equilibrium within 1 min. As this is a steady-state approach, fluorescence polarization measurement was taken after incubation for 5 min for each corresponding concentrations of the protein and drug to ensure that the binding has reached equilibrium. It should be noted that the detergent concentration was kept constant at all times to eliminate the change in polarization generated by drug–DDM micelle interaction. All measurements were performed at 25 °C using a PerkinElmer LS55 spectrofluorometer equipped with a Hamamatsu R928 photomultiplier. The excitation wavelengths were 527, 483, 447, and 330 nm, respectively, for R6G, Et, Pf, and Cip. Fluorescence polarization signals (in ∆P) were measured at emission wavelengths of 550, 620, 508, and 415 nm, respectively, for these ligands. Each titration point recorded was an average of 15 measurements. Data were analyzed using the equation, 

\[ P = \frac{(P_{\text{bound}}-P_{\text{free}})\text{[protein]}}{K_D + \text{[protein]}} + P_{\text{free}} \]

where \( P \) is the polarization measured at a given total protein concentration, \( P_{\text{free}} \) is the initial polarization of free ligand, \( P_{\text{bound}} \) is the maximum polarization of specifically bound ligand, and \([\text{protein}]\) is the protein concentration. The titration experiments were repeated for three times to obtained the average \( K_D \) value. Curve fitting was accomplished using the program ORIGIN [21].

3. Results

3.1. Binding affinities of various AcrB ligands

The goal of this research was to determine the binding affinities of a variety of ligands to AcrB. Fluorescence polarization based-assay was initially carried out to study the interaction between AcrB and R6G. (Fig. 1a) illustrates the binding isotherm of AcrB in the presence of 1 µM R6G. As presented in the figure, a simple hyperbolic curve was observed with the \( K_D \) of 5.5 ± 0.9 µM. The Hill plot of the data (Fig. 1b) yields a Hill coefficient of 1, suggesting a simple drug binding process with no cooperativity.

Fluorescence polarization was also used to observe the binding of Et, Pf and Cip to the transporter. In comparison with R6G, other ligands tested show somewhat lower affinity.
Thus, Et bound with a $K_D$ of 8.7 ± 1.9 µM (Fig. 2a), Pf with that of 14.5 ± 1.1 µM (Fig. 2b), and Cip with that of 74.1 ± 2.6 µM (Fig. 2c).

3.2. Binding of two drugs by AcrB

We carried out several titration experiments that involved the initial saturation of AcrB with 100 µM of Et. To ensure that AcrB and Et formed a complex, the mixture was incubated for at least 2 h before titrating with 1.5 µM Pf. Fluorescence polarization experiments indicated that AcrB-Et binds Pf with a $K_D$ of 61.0 ± 0.9 µM (Fig. 3). This value is about four times of the $K_D$ of Pf (14.5 ± 1.1 µM) in the absence of Et, indicating that Et interferes with the binding of Pf to AcrB, possibly because the two compounds compete with each other for the same binding site.

We carried out similar titration experiments with the pre-formed AcrB–Et complex using 1.5 µM Cip. The results gave a $K_D$ value of 70.4 ± 15.4 µM for Cip (data not shown), which is not significantly different from the $K_D$ of Cip (74.1 ± 2.6 µM) determined in the absence of Et. Although it is tempting to conclude that Et and Cip bind to non-overlapping sites, the numerical precision of $K_D$ values is not high in this case because of the quite low affinity of this ligand, and we believe that further experiments utilizing different approaches are needed in this case.

3.3. pH dependence of drug binding to AcrB

We performed fluorescence polarization experiments to determine the binding of R6G to AcrB at different pH values. Fig. 4 illustrates the decrease in $K_D$ values for rhodamine 6G as the pH increases from 5.5 to 8.4.

3.4. Detergent concentration does not affect drug binding to AcrB

To analyze if the detergent concentration affects the fluorescence polarization results, we measured the dissociation constant of the AcrB–R6G complex in different concentrations of detergent, DDM. We found that at moderate detergent concentrations, the concentration of...
detergent does not have much influence on the results. At 0.5%, 0.05%, and 0.005% DDM, the dissociation constants of the AcrB–R6G complex were $7.2 \pm 0.1$, $5.5 \pm 0.9$, and $4.4 \pm 0.8 \mu\text{M}$, respectively.

4. Discussion

We demonstrated that the technique of fluorescence polarization is sensitive and precise enough to detect ligand binding of *E. coli* AcrB, an intrinsic membrane protein in a detergent environment. As this is a steady-state approach, it is important to ensure that the binding equilibrium has reached before data collection. A stop-flow study of an oligopeptide OppA transporter, belonging to the ABC superfamily, indicated that peptide binding to OppA would reach equilibrium within 20 ms when the concentrations of the protein and peptide are in the micromolar range [22]. Thus, a 5 min period for incubation in our experiments should be more than enough to allow the binding to reach equilibrium. We obtained results showing that the ligands bind to AcrB with their $K_D$ values between $5.5$ (R6G) and $74.1 \mu\text{M}$ (Cip) (Fig. 1 and Fig. 2). These values are similar to the $K_D$ for most substrates for the MdfA (a MFS transporter) [23] and EmrE (a SMR transporter) [24] multidrug pumps, determined by competition with tetr phenylphosphonium binding. Although our data suggested the binding stoichiometry of 1:1 monomeric AcrB-to-drug molar ratio, there is a formal possibility that any compound may bind to more than one site on the protein with similar affinity that could not be discriminated using this technique. Thus, further experiments utilizing different approaches are needed to confirm this drug binding stoichiometry.

One of the main goals of our work is to introduce a relatively simple approach to study membrane protein–ligand interaction in a detergent environment. It should be note that detergent solubilized protein in detergent micelles may behave differently from that reconstituted in a lipid environment, including altering the binding affinity of drugs to AcrB in this case. However, a study of the EmrE transporter suggested that EmrE binds drugs with similar strengths in different membrane mimetic environments, including those in DDM micelles and in reconstituted lipid vesicles [25]. The observed dissociation constants were in the micromolar range for the EmrE drugs in any of the membrane mimetic environments
Detailed study of AcrB–ligand interaction in the lipid environment would be necessary to determine whether the reconstituted AcrB behaves differently in terms of drug binding affinity.

As our fluorescence polarization assay was performed in a detergent environment, we are aware of the fact that detergent micelles in solution may have interaction with the drug molecules. To eliminate this “non-specific” binding component that may cause an error for the measurement of drug binding affinity, it is important to keep the detergent concentration constant during titrations. It has been reported that the dissociation constants of DDM micelle with Et and Pf are 5.2 and 17.1 mM, respectively [25]. We measured the binding affinities of DDM micelle with R6G and Cip using fluorescence polarization assay (data not shown). We found that the interactions between the micelles and drugs are quite weak. Thus, we could only estimate these $K_D$ values, which were 2.9 mM for R6G–DDM micelle and 899.7 mM for Cip–DDM micelle, respectively. Apparently, the drug–DDM micelle interactions are in the millimolar range, which are about three orders of magnitude weaker than those of the drug–transporter interactions.

We also examined the question of simultaneous binding of two different drugs to AcrB, by using fluorescence polarization assays. Et was a convenient ligand because it absorbs and emits light at very long wavelengths, far away from the wavelengths used by other dyes. Titrations of Pf into Et-saturated AcrB yielded a much increased dissociation constant compared with that in the absence of Et, suggesting strongly that both drugs bind in a competitive manner to an overlapping binding site in AcrB.

To confirm that two drugs compete with one another for a specific binding site in the protein, we performed competition experiments in which tetraphenylphosphonium chloride (TPP) was titrated into a solution containing the preformed AcrB–R6G complex. In this case, TPP was chosen as a second ligand to knock off the bound R6G from AcrB. The absorption spectra of TPP (from 200 to 600 nm) showed that this molecule absorbs light at the wavelengths of 224.9, 268.0 and 275.9 nm. At $\lambda = 527$ nm, which is the excitation wavelength for R6G, the energy is too low to excite TPP. Thus, TPP was treated as a non-
fluorescent ligand in the “knock off” experiments. The data revealed that TPP was able to bind AcrB and replace the bound R6G molecule from the protein as demonstrated by the release of R6G that resulted in the reduction of polarization (Fig. 5). This binding assay provides direct evidence that TPP interferes with the binding of R6G. However, it is not known that this heterologous displacement is due to a truly competitive binding or via negative heterotropic allostery between distinct binding sites of the ligands. We were unable to determine the binding affinity of TPP through these competition experiments due to precipitation of the protein at higher TPP concentrations. Regardless, the titrations demonstrate that R6G is bound specifically in the AcrB transporter.

We also tried these competition experiments using erythromycin, which is another AcrB drug, as a second ligand to replace the bound R6G from the transporter (not shown). The data revealed that erythromycin is not capable of knocking off the bound R6G, as the polarization of R6G does not change during titrations. Thus, there is a chance that different class of drugs may bind at different sites in AcrB.

The binding of R6G to AcrB was strongly pH-dependent. A trivial explanation of this result was the pH-dependent alteration of charge state in the ligand. However, we found that there is essentially no change in the emission spectra of free R6G in the pH range examined in Fig. 4, suggesting that the electronic arrangement of the protonated and de-protonated states of R6G are very similar. The pKₐ of R6G is nearly neutral, with the value equals 7.5 [26] (the pKₐs of Pf and Cip are 8.1 and 6.1, respectively [27]). At pH < pKₐ, R6G should exist in its protonated form. When pH > pKₐ, this dye should be predominantly unionized. It is expected that R6G should bind tighter to the transporter at acidic pH, as it is predominantly in the positively charged form. However, the reverse case is seen from the pH dependent data. One possible explanation is that R6G possesses a large degree of delocalization. It is this large extent of electron delocalization that makes the chemical properties of the protonated and de-protonated forms of R6G very similar. Indeed, it has been reported that the observed octane/water partition coefficient of R6G remains the same from pH 4 to 11 because of this charge delocalization phenomenon [26].
Judging from the binding isotherms of AcrB in the presence of 1 µM R6G, these curves maintained the simple hyperbolic shape and reached saturation at the same AcrB concentration at different pH values (as shown in Fig. 1a). Thus, it is not likely that altering the pH affects the capacity of binding. Perhaps the pH effect is related to the protonation state of acidic residues in the binding pocket: there are several acidic residues in the ligand binding site of the asymmetric AcrB crystal [12], with Asp276 particularly close to the ligand. The strong pH dependence for drug binding has been observed as the result of binding of cationic drugs with the glutamate residue in the EmrE multidrug transporter [24]. Alternatively, it could involve global conformational changes of the transporter that utilizes the transmembrane electrochemical gradient of protons for activity.

Fluorescence polarization assay has been widely used for studying protein–DNA interactions [19], [20], [28] and [29], and lately for determining affinities of drug binding in transcriptional regulators [29], [30] and [31]. To our knowledge, this is the first attempt using this methodology to investigate interaction between membrane protein and bound drug. The availability of fluorescence polarization to measure affinities of a variety of AcrB substrates will provide the means for studying the interactions of other purified transporters with their substrates in detergent solutions.

5. References


[21] ORIGIN Ver. 7.5. OriginLab Corporation, Northampton, MA, USA.


Fig. 1. Representative fluorescence polarization of AcrB in 0.05% DDM with R6G. (a) Binding isotherm of AcrB with R6G, showing a $K_D$ of $5.5 \pm 0.9$ µM, in buffer containing 20 mM Tris (pH 7.5) and 0.05% DDM. (b) Hill plot of the data obtained for R6G binding to AcrB. $\alpha$ corresponds to the fraction of bound R6G. The plot gives a slope of $1.12 \pm 0.02$, indicating a simple binding process with a stoichiometry of one AcrB protomer per one drug molecule. The interception of the plot provides a $K_D$ of $5.4 \pm 1.0$ µM for the R6G binding.
Fig. 2. Fluorescence polarization of AcrB with Et, Pf and Cip. Binding isotherms of AcrB with (a) Et, showing a $K_D$ of $8.7 \pm 1.9 \, \mu$M, (b) Pf, showing a $K_D$ of $14.5 \pm 1.1 \, \mu$M, and (c) Cip, showing a $K_D$ of $74.1 \pm 2.6 \, \mu$M.
Fig. 3. Binding of ligands by AcrB in the presence of 100µM of Et as determined by fluorescence polarization assay. The change in fluorescence polarization signals (ΔFP) of Pf was measured at an emission wavelength of 508 nm. The binding curve suggests a $K_D$ of 61.0 ±0.9 µM for Pf.
Fig. 4. Effect of pH on the $K_D$ of R6G binding to AcrB. The resulting $K_{DS}$ were plotted against pH.
Fig. 5. AcrB binding competition experiment between R6G and TPP. AcrB (10µM) was pre-incubated with R6G (1µM) for 2h before titration. The change in fluorescence polarization signals (ΔFP) of R6G was measured at an emission wavelength of 550nm. TPP was non-fluorescent in the experimental conditions. The decrease in ΔFP showed that the bound R6G was knocked off by TPP.
CHAPTER 4. Characterization of the multidrug efflux regulator AcrR from

*Escherichia coli*

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Abstract

The *Escherichia coli* AcrR represses transcription of the *acrB* gene, which encodes the multidrug efflux pump AcrB that extrudes a wide variety of toxic compounds, by binding its target operator DNA. Fluorescence polarization was performed using purified, recombinant AcrR that contains a 6xHis tag at the C-terminus and a fluorescein-labeled 28-base pair oligonucleotide bearing a predicted palindrome (IR) operator sequence. Binding of AcrR to the predicted IR sequence occurred with a dissociation constant ($K_D$) in the nanomolar range. Fluorescence polarization assays were also applied to characterize the affinity and specificity of AcrR interaction with three different fluorescent ligands, rhodamine 6G, ethidium, and proflavin. The $K_D$ values for these ligands range from 4.2 to 10.1 µM, suggesting that AcrR is capable of recognizing a wide range of structurally dissimilar toxic compounds as it is in the case of the AcrB multidrug efflux pump. We found that the binding of rhodamine 6G to AcrR is inhibited by the presence of ethidium. In contrast, the dissociation constant of proflavin binding to AcrR was not affected by ethidium, a result suggesting that ethidium and proflavin are bound to distinct binding sites.
Introduction

The increase in bacterial resistance to multiple drugs has emerged as a major clinical problem. One important mechanism that gives rise to multidrug resistance (MDR) in bacteria is the expression of multidrug transporters that are often regulated at the transcriptional level by transcriptional regulators [1]. Many of these transcriptional regulators are multi-drug binding proteins, which recognize the same array of toxic chemicals extruded by the transporters that they regulate [2]. These regulators often act as cytosolic chemical sensors and respond to threatening levels of toxic chemicals. The results are the over-expression of MDR transporters, which, in turn, promote efflux from cells, thus protecting them from toxic substances.

Bacterial efflux transporters capable of transporting multiple toxic compounds fall into five classes: (i) the ATP-binding cassette (ABC) family [3]; (ii) the major facilitator superfamily (MFS) [4] and [5]; (iii) the resistance-nodulation-division (RND) family [6] and [7]; (iv) the small multidrug resistance (SMR) family [8]; and (v) the multidrug and toxic compound extrusion (MATE) family [9]. The ABC family transporters take the free energy generated from ATP hydrolysis to expel toxic substances out of cells [10]. Efflux transporters in the other families, however, utilize the transmembrane electrochemical gradient of protons or sodium ions to extrude these harmful substrates from cells [10].

Of all known MDR transporters, the *Escherichia coli* AcrB multidrug efflux pump, which belongs to the RND family, shows the widest substrate specificity ranging from most of the current use antibiotics, disinfectants, dyes, bile salts, fatty acids, and detergents, to simple solvents [11]. AcrB is regulated by a global transcriptional activator MarA and a local transcriptional repressor AcrR [12]. The *acrR* gene is located 141 bp upstream of the *acrAB* operon and transcribed divergently [12]. It encodes a 215-amino-acid protein, which shares N-terminal sequence and structural similarities to members of the TetR family of transcriptional repressors [13]. Like other members in the TetR family, the N-terminal domain of AcrR contains a predicted DNA-binding helix-turn-helix (HTH) motif, while its
C-terminal domain has unique sequences and is expected to form a multi-drug binding site for its inducing drugs.

As a member of the TetR family of transcriptional regulators, it is expected that binding of drug to the C-terminal ligand-binding domain of AcrR triggers conformational change in its N-terminal DNA-binding region. This change in conformation results in the release of AcrR from its operator DNA, and thus allows transcription from its cognate promoter.

At present, the AcrR palindrome (IR) operator site has not been identified. However, there is strong evidence that this IR is composed of a 24 bp sequence, 5'TACATACATTTTGAAATGTATGTA3'. Ma et al. [12] demonstrated that AcrR interacts with the acr promoter located in the section between acrR and acrAB. A comparison of nucleotide sequences upstream of multidrug resistance transport genes also predicted that the upstream region of the acrAB operon contains a candidate IR operator. This operator consists of an inverting 24 bp sequence, which is well conserved and overlaps with the acrAB promoter [14].

In this study, we used fluorescence polarization assay [15], [16] and [17] to examine interaction between AcrR and its predicted target DNA. We also used this method to determine the affinities of three different AcrR drugs, ethidium (Et), proflavin (Pf), and rhodamine 6G (R6G). So far, all known members of the TetR family have been reported to bind target DNAs in the form of homodimers as basic units [13]. Thus, the DNA-binding data were fitted using an AcrR dimer as a fundamental unit. For drug binding, we fitted these data using a monomeric AcrR as a single structural unit since many of the TetR family members bind ligands in both subunits of the homodimers, although QacR only binds one drug molecule in the dimer [18]. The possibility of simultaneous binding of two different drugs by AcrR was also examined using this fluorescence polarization technique.
Results

DNA binding

Presumably, AcrR suppresses the expression of the AcrB multidrug efflux pump by binding its target DNA. As mentioned above, the predicted IR operator of AcrR is composed of an inverting 24 bp sequence. Fluorescence polarization-based assay was carried out to study the interaction between AcrR and a 28 bp DNA containing the IR sequence. Fig. 1A illustrates the binding isotherm of AcrR in the presence of 1 nM fluoresceinated DNA. The titration experiment indicated that AcrR binds the 28 bp operator, with a dissociation constant, $K_D$, of 20.2 ± 1.4 nM. This value is substantially lower than those of the CmeR ($K_D = 88$ nM) [19] and EthR ($K_D = 146$ nM) [20] repressors, but albeit higher than the values of TetR ($K_D = 0.2$ nM) [21], MtrR ($K_D = 0.9$ nM) [17], and QacR ($K_D = 5.7$ nM) [17] in the TetR family. It should be noted that this $K_D$ is sensitive to the concentration of salts in titration buffers, and in some cases also the length of oligodeoxynucleotides. Doubling the salt concentration may increase the $K_D$ by two orders of magnitude as shown in MtrR [17].

A Hill plot of the DNA-binding data yielded a Hill coefficient of 2 (Fig. 1B), suggesting a stoichiometry of two AcrR dimers per cognate DNA. The corresponding Scatchard plot showed a typical convex curve (see Suppl. Fig. S1), a result suggesting that more than one dimer of AcrR bind cooperatively to the DNA. To confirm the stoichiometry of AcrR and DNA binding, the protein was titrated into the binding buffer (containing 500 nM fluoresceinated DNA) until the concentration of AcrR (as dimer) reached 1500 nM (Fig. 1C). The result indicated that the inflection point occurs at a dimeric AcrR concentration of 1000 nM, suggesting a stoichiometry of two dimers of AcrR per cognate DNA.

Binding of AcrR ligands

The goal of this experiment was to determine the binding affinities of a variety of AcrR ligands. We initially measured the binding affinity of Et by AcrR. Fluorescence
polarization measurements showed that the $K_D$ of the AcrR–Et complex is $4.2 \pm 0.3 \, \mu M$ (Fig. 2A). The Hill and Scatchard plots of the data (Fig. 2B and C) suggested that the protein employs a simple binding stoichiometry of 1:1 monomeric AcrR to Et molar ratio.

Fluorescence polarization was also used to observe the Pf and R6G binding (Fig. 3). The values of $K_D$ were found to be $10.1 \pm 0.8 \, \mu M$ and $10.7 \pm 1.4 \, \mu M$, respectively. QacR binds Et, Pf, and R6G with $K_D$ values of 186, 42, and $0.8 \, \mu M$, respectively [22]. Thus, AcrR appears to have stronger interaction with Et and Pf, but weaker with R6G.

**Evidence of simultaneous binding of two drugs by AcrR**

It has been shown that two different drugs bind simultaneously to two different sites in a large binding pocket of the QacR regulatory protein [22]. This raises the question whether AcrR can accommodate two different drugs at a time. We carried out a series of titration experiments that involved the initial saturation of AcrR with $100 \, \mu M$ of Et. To ensure that AcrR and Et formed a complex, the mixture of AcrR and Et was incubated for at least two hours before titration. The pre-formed AcrR–Et complex was then titrated with $1 \, \mu M$ of Pf. Fluorescence polarization assay was used to monitor the change in polarization of Pf. By using this method, we were able to provide evidence for the simultaneous binding of two drugs by AcrR. The polarization experiments indicated that AcrR binds Pf, with a $K_D$ of $14.9 \pm 0.9 \, \mu M$, in the presence of $100 \, \mu M$ Et. Fig. 4A depicts the binding isotherm of the AcrR–Et complex in titrating with Pf. This value is very close to the $K_D$ of Pf ($10.1 \pm 0.8 \, \mu M$) in the absence of Et, indicating that the pre-bound Et does not affect the binding of Pf to the regulator.

We also studied the binding of R6G in the presence of $100 \, \mu M$ Et using the same method. Fluorescence polarization experiments indicated that AcrR–Et binds R6G with a $K_D$ of $132.0 \pm 9.1 \, \mu M$ (Fig. 4B). This $K_D$ is about 13 times of that of R6G in the absence of Et, suggesting the presence of Et severely interferes with the binding of R6G in AcrR.
Discussion

Using recombinant AcrR protein that contains a 6xHis tag at the C-terminus, we confirmed that AcrR interacts with the 28 bp DNA, 5′TTTACATACATTGTGAATGTATGTACC3′. This DNA contains the inverting 24 bp IR. IR is a typical DNA sequence that forms binding sites for regulatory proteins in the promoter regions. A gel mobility shift assay, using a DNA fragment bearing the sequence of the promoter region between acrR and acrAB, has demonstrated that AcrR binds to the promoter sequence. Thus, our experimental data strongly suggest that this predicted 24 bp IR, located at the promoter region of acrR–acrAB, is very likely to serve as the acr operator.

The affinity of AcrR for IR is in the nanomolar range (20.2 nM), and this dissociation constant is closer to that of QacR among members of the TetR family. The IR sequence of AcrR is similar in length to that of the long 28 bp IR1 recognized by QacR, but is different from the 15 bp tetO bound by TetR. TetR binds as a single dimer to the tetO operator [23], however, two dimers of QacR interact with one IR1 [24]. Based on the length of the IR sequence, we reasoned that AcrR might bind its operator as two homodimers in a way that is similar to the QacR–DNA binding. Our fluorescence polarization measurements suggested that two AcrR dimers interact with one 28 bp IR. Thus, it is very likely that two homodimers of AcrR bind the 24 bp target DNA cooperatively as shown in the structure of the QacR–DNA complex [24].

The affinities of AcrR to its drugs are in the micromolar region, and the dissociation constants are quite similar to those of QacR [25], BmrR [26], and TtgV [27]. Based on the fluorescence polarization assays, titrations of AcrR to the ligand-binding solutions give a stoichiometry of 1:1 monomeric AcrR-to-drug molar ratio. As the recent preliminary X-ray diffraction data suggest a dimeric assembly of the AcrR regulator [28], thus we believe that each C-terminal ligand-binding site of AcrR (each subunit of which contains one site) in the homodimer binds a drug molecule. This drug-binding mode is the same as that of TetR [29], but different from the ligand-binding mode of QacR which binds one drug molecule per homodimer [22]. In comparing the DNA and drug-binding modes of AcrR with those of
TetR and QacR, AcrR is distinct in that its DNA-binding mode is similar to that of QacR, however, it employs the mode of drug-binding similar to that of TetR. Thus, the induction mechanism of AcrR may have unique features that have not been revealed by previous studies concerning other regulators in the TetR family.

To address the question whether AcrR allows simultaneous binding of two different drugs, we used a ligand-binding assay that utilizes the polarization of fluorescence. Our experimental results suggest that AcrR is most likely to bind Et and R6G competitively as the binding affinity of R6G is greatly decreased in the presence of Et. In the case of Et and Pf, Pf is bound by both apo-AcrR and AcrR–Et in equal affinity, indicating that Et and Pf may exhibit noncompetitive binding. If this is the case, it is anticipated that Et and Pf are bound to distinct and unoverlapped binding sites in the regulator. Indeed, a docking study using the most recent crystal structure of apo-AcrR determined in our laboratory suggests that Et and Pf bind independently at different places in the large multi-drug binding pocket of AcrR (see Suppl. Fig. S4). This simultaneous drug-binding phenomenon has been found in the MdfA transporter [30] and the recent study QacR repressor [22]. Thus, it is not surprising that AcrR is capable of accommodating two different drugs at a time.

Materials and methods

Expression and purification of recombinant AcrR. The cloning, expression, and purification of recombinant AcrR that contains a 6xHis tag at the C-terminus were done as described before [28]. The purified 6xHis AcrR protein was extensively dialyzed against buffer containing 10 mM Na-phosphate (pH 7.2) and 100 mM NaCl, and concentrated to 10 mg/ml.

Fluorescence polarization assay for the DNA-binding affinity. Fluorescence polarization assays were used to determine the DNA-binding affinities of the AcrR regulator. Both the 28 bp oligodeoxynucleotide and fluorescein-labeled oligodeoxynucleotide were purchased from IDT, Inc. (Coralville, IA). These oligodeoxynucleotides contain the predicted 24 bp IR site for AcrR binding. Their sequences were 5′-
TTTACATACATTGTGAATGTATGTACC-3’ and 5’-F-GGTACATACATTCACAAATGTATGTAAA-3’, where F denotes the fluorescein which was covalently attached to the 5’ end of the oligodeoxynucleotide by a hexamethylene linker. The 28 bp fluoresceinated ds-DNA was prepared by annealing these two oligodeoxynucleotides together. Fluorescence polarization experiment was done using a DNA-binding solution containing 10 mM Na-phosphate (pH 7.2), 100 mM NaCl, 1 nM fluoresceinated DNA, and 1 µg of poly(dI–dC) as non-specific DNA. The protein solution containing 500 nM dimeric AcrR and 1 nM fluoresceinated DNA was titrated into the DNA-binding solution until the millipolarization (mP) become unchanged. All measurements were performed at 25 °C using a PerkinElmer LS55 spectrofluorometer equipped with a Hamamatsu R928 photomultiplier. The excitation wavelength was 490 nm, and the fluorescence polarization signal (in ∆P) was measured at 520 nm. Each titration point recorded was an average of 15 measurements. Data were analyzed using the equation,

\[ P = \left\{ \frac{(P_{\text{bound}} - P_{\text{free}}) [\text{protein}]^2}{K_D^2 + [\text{protein}]^2} \right\} + P_{\text{free}}, \]

where \( P \) is the polarization measured at a given total protein concentration, \( P_{\text{free}} \) is the initial polarization of free fluorescein-labeled DNA, \( P_{\text{bound}} \) is the maximum polarization of specifically bound DNA, and \([\text{protein}]\) is the protein concentration. The titration experiments were repeated for three times to obtained the average \( K_D \) value. Curve fitting was accomplished using the program ORIGIN [31].

**Fluorescence polarization assay for AcrR ligand-binding affinities.** The experimental procedures for determining ligand-binding affinities of AcrR using the technique of fluorescence polarization were similar to that of the DNA-binding assay. The experiments were done using a ligand-binding solution containing 10 mM Na-phosphate (pH 7.2), 100 mM NaCl, and 1 µM ligand (R6G, Et, or Pf). The AcrR protein solution in 10 mM Na-phosphate (pH 7.2), 100 mM NaCl, and 1 µM ligand was titrated into the ligand-binding solution until the polarization (\( P \)) become unchanged. The excitation wavelengths were 527, 483, and 447 nm, respectively, for R6G, Et, and Pf. Fluorescence polarization signals (in ∆P) were measured at emission wavelengths of 550, 620, and 508 nm, respectively, for these ligands. Each titration point recorded was an average of 15 measurements. The titration
experiments were repeated for three times to obtain the average $K_D$ values. Curve fitting was accomplished using the program ORIGIN [31].

References


[31] ORIGIN Ver. 7.5. OriginLab Corporation, Northampton, MA, USA.
Fig. 1. AcrR binding to the 28-mer cognate oligodeoxynucleotide. (A) Binding isotherm of AcrR in the presence of 10 mM Na-phosphate (pH 7.2), 100 mM NaCl, 1 nM fluoresceinated DNA, and 1 µg of poly(dI–dC) as non-specific DNA. The sequence of one of the IR strands is 5′-TTTACATACTTGTGAATGTATGTACC-3′. The binding curve suggests a $K_D$ of 20.2 ± 1.4 nM. (B) Hill plot of the data obtained for IR binding to AcrR. $\alpha$ corresponds to the fraction of bound IR. The plot gives a slope of 2.06 ± 0.12, indicating a cooperative binding process with a stoichiometry of two AcrR dimers per one IR. The interception of the plot provides a $K_D$ of 19.2 ± 1.0 nM for the IR binding. (C) Determination of the stoichiometry of AcrR–DNA binding. The inflection point at an AcrR dimer concentration of 1000 nM in the presence of 500 nM DNA, indicating the stoichiometry of 2:1 protein-to-DNA ratio.
Fig. 2. Representative fluorescence polarization of AcrR with Et. (A) Binding isotherm of AcrR with Et, showing a $K_D$ of $4.2 \pm 0.3 \mu M$. (B) Hill plot of the data obtained for Et binding to AcrR. $\alpha$ corresponds to the fraction of bound Et. The plot gives a slope of $1.02 \pm 0.02$, indicating a simple binding process with a stoichiometry of one AcrR protomer per one drug molecule. The interception of the plot provides a $K_D$ of $4.4 \pm 1.0 \mu M$. (C) Linearization of the data obtained for Et binding to AcrR in a Scatchard plot, indicating a $K_D$ of $4.6 \pm 0.3 \mu M$. 
Fig. 3. Fluorescence polarization of AcrR with Pf and R6G. (A) Binding isotherm of AcrR with Pf, showing a $K_D$ of 10.1 ± 0.8 µM. (B) Hill plot of the data obtained for Pf binding to AcrR. $\alpha$ corresponds to the fraction of bound Pf. The plot gives a slope of 1.12 ± 0.05, and the interception provides a $K_D$ of 9.6 ± 1.1 µM. (C) Binding isotherm of AcrR with R6G, showing a $K_D$ of 10.7 ± 1.4 µM. (D) Hill plot of the data obtained for R6G binding to AcrR. $\alpha$ corresponds to the fraction of bound R6G. The plot gives a slope of 1.14 ± 0.09, and the interception provides a $K_D$ of 11.1 ± 1.2 µM.
Fig. 4. Binding of ligands by AcrR in the presence of 100 µM of Et as determined by fluorescence polarization assay. (A) The change in fluorescence polarization signals (ΔFP) of Pf measured at an emission wavelength of 508 nm. The binding curve suggests a $K_D$ of 14.9 ± 0.9 µM for Pf. (B) The change in fluorescence polarization signals (ΔFP) of R6G measured at an emission wavelength of 550 nm. The binding curve suggests a $K_D$ of 132.7 ± 14.6 µM for R6G. The maximum concentration of the AcrR monomer was 50 µM.
CHAPTER 5. Crystal Structure of the Transcriptional Regulator CmeR from *Campylobacter jejuni*

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Abstract

The CmeABC multidrug efflux pump, which belongs to the resistance-nodulation-division (RND) family, recognizes and extrudes a broad range of antimicrobial agents and is essential for *Campylobacter jejuni* colonization of the animal intestinal tract by mediating the efflux of bile acids. The expression of CmeABC is controlled by the transcriptional regulator CmeR, whose open reading frame is located immediately upstream of the *cmeABC* operon. To understand the structural basis of CmeR regulation, we have determined the crystal structure of CmeR to 2.2 Å resolution, revealing a dimeric two-domain molecule with an entirely helical architecture similar to members of the TetR family of transcriptional regulators. Unlike the rest of the TetR regulators, CmeR has a large center-to-center distance (54 Å) between two N termini of the dimer, and a large flexible ligand-binding pocket in the C-terminal domain. Each monomer forms a 20 Å long tunnel-like cavity in the ligand-binding domain of CmeR and is occupied by a fortuitous ligand that is identified as glycerol. The binding of glycerol to CmeR induces a conformational state that is incompatible with target DNA. As glycerol has a chemical structure similar to that of potential ligands of CmeR, the structure obtained mimics the induced form of CmeR. These findings reveal novel structural features of a TetR family regulator, and provide new insight into the mechanisms of ligand binding and CmeR regulation.
Introduction

*Campylobacter jejuni* is the leading bacterial cause of food-borne diarrhea in the USA and other developed countries. It is also a significant enteric pathogen for young children in developing countries. This Gram-negative enteric organism colonizes the intestinal tracts of animals and has become increasingly resistant to antimicrobials due to the possession of multidrug efflux transporters and acquisition of various resistance mechanisms, compromising the effectiveness of antibiotic treatment. According to the genomic sequence of NCTC 11168, *C. jejuni* harbors 13 putative antibiotic efflux transporters of the ATP-binding cassette (ABC), resistance-nodulation-division (RND), multidrug and toxic compound extrusion (MATE), major facilitator (MF), and small multidrug resistance (SMR) families. At present, CmeABC and CmeDEF, which belong to the RND family, are the only two antibiotic efflux transporters that have been functionally characterized in *Campylobacter*.

The CmeABC efflux system is the main efflux pump in *C. jejuni* and consists of three members, including an outer membrane channel (CmeC), an inner membrane drug transporter (CmeB), and a periplasmic membrane fusion protein (CmeA). These three proteins are encoded by a three-gene operon (*cmeABC*) and form an efflux system that extrudes a variety of toxic compounds directly out of *C. jejuni*. The substrates extruded by CmeABC include commonly used antibiotics (e.g. fluoroquinolones, macrolides, ampicillin, tetracycline, chloramphenicol, cefotaxime, rifampin), metal ions (e.g. Co$^{2+}$ and Cu$^{2+}$), and lipophilic compounds (e.g. SDS and various bile salts). Thus, CmeABC contributes significantly to the intrinsic and acquired resistance of *Campylobacter* to structurally diverse antimicrobials. In addition, this efflux system is essential for *Campylobacter* colonization in the animal intestinal tract by conferring resistance to the bile acids that are normally present in the animal intestinal tract and have bactericidal effect.

The expression of *cmeABC* is controlled by the transcriptional regulator CmeR. The *cmeR* gene is located immediately upstream of the *cmeABC* operon and encodes a 210 amino acid residue protein that shares N-terminal sequence and structural similarities with members of the TetR family of transcriptional repressors. Like other members of the TetR family, the N-terminal domain of CmeR contains a predicted DNA-binding helix-turn-helix (HTH)
motif, while its C-terminal region has unique sequences and is expected to be involved in the binding of inducing ligands.\textsuperscript{10,11} cmeR is transcribed in the same direction as cmeABC, and the intergenic region between cmeR and cmeA contains the 16 bp inverted repeat (IR) operator site for cmeABC. As a transcriptional regulator, CmeR binds directly to the IR operator and represses the transcription of cmeABC.\textsuperscript{10} This regulating process is similar to those of the other TetR family members, such as AcrR of \textit{Escherichia coli},\textsuperscript{13} MexR of \textit{Pseudomonas aeruginosa},\textsuperscript{14} MtrR of \textit{Neisseria gonorrhoeae},\textsuperscript{15} and QacR of \textit{Staphylococcus aureus},\textsuperscript{16} in which they bind specifically to the promoter sequences of acrAB, mexAB, mtrCDE, and qacA, respectively, thus inhibiting the expression of the corresponding efflux pump(s). Deletion of cmeR or mutations in the IR operator releases the repression, resulting in the over-expression of CmeABC, which, in turn, leads to the enhanced resistance to multiple antibiotics.\textsuperscript{10}

In addition, bile compounds, including both conjugated (e.g. taurodeoxycholate) and non-conjugated (e.g. cholate), induce the expression of cmeABC by inhibiting the binding of CmeR to the promoter of cmeABC, suggesting that bile compounds are inducing ligands of CmeR.\textsuperscript{17} It is possible that CmeR can be induced by other unidentified ligands. How inducing ligands bind to CmeR and modulate the expression of CmeR-controlled genes is not known. On the basis of the predicted structural features, we hypothesize that binding of inducing ligands to the C-terminal domain of CmeR triggers conformational change in the N-terminal DNA-binding region. This change in conformation results in the release of CmeR from its operator DNA, and thus allows transcription from its cognate promoter. As an initial step to examine this hypothesis and elucidate the mechanisms that CmeR uses to regulate gene expression, we present here the crystal structure at 2.2 Å resolution of the CmeR regulator.
Results

Overall structure of CmeR

We used the multiple-wavelength anomalous dispersion method to solve the selenomethionyl-substituted (SeMet) CmeR crystal structure from *C. jejuni*. Its native crystal structure was then determined to 2.2 Å resolution (Table 1 and Figure 1(a)), revealing that only one CmeR molecule exists in the asymmetric unit. The dimeric arrangement of the protein was found by applying the crystallographic symmetry operators.

The dimeric structure of CmeR, indicating an all-helical protein, is shown in Figure 1(b). As a member of the TetR family of transcriptional regulators, CmeR consists of two functional motifs; an N-terminal DNA-binding domain and a C-terminal ligand-binding domain.\(^{11,12}\)

The crystal structure revealed that each subunit of CmeR is composed of ten α helices (α1–α10 and α1′–α10′, respectively) and indeed can be divided into two domains. The smaller N-terminal domain shares considerably high levels of sequence and structural similarities with the other TetR family members. For example, residues 12–65 possess 23% amino acid identity with and 43% similarity to that of TetR.\(^ {18}\) This N-terminal region also shows 50% identity with and 71% homology to that of the QacR repressor.\(^ {19}\) Among the TetR family, including TetR,\(^ {18,20}\) QacR,\(^ {19,21}\) CprB,\(^ {22}\) and EthR,\(^ {23,24}\) the N-terminal domains of these transcriptional regulators are formed by three α-helix bundles. The structure of CmeR, however, revealed that the third short α-helix, presumably formed by residues 47–53 is missing. Instead, these residues form a random coil with noticeably high B-factors, suggesting a mobile nature of this coil. To facilitate the comparison with the structures of other TetR members, helices of CmeR are numbered from the N terminus as α1 (7-29), α2 (36-43), α4 (57-81), α5 (88-104), α6 (106-118), α7 (a(125-136) and b(138-148)), α8 (152-170), α9 (172-180), and α10 (187-203), in which helix α3 has been skipped. In this arrangement, the larger C-terminal domain comprises seven α helices (α4–α10) and is involved in the dimerization of the repressor. According to the ligand-bound structures of
TetR, QacR, and EthR, the C-terminal region also forms the drug-binding domain. Like TetR, QacR and EthR, the crystal structure of CmeR suggests that helices 4–9 form the ligand-binding domain of the regulator.

**N-terminal domain**

The overall structure of the N-terminal DNA-binding domain of CmeR is quite similar to those of the TetR family members. A superposition of \( \alpha \) atoms, between residues 14 and 44, of CmeR with their corresponding residues in QacR gives an overall rmsd of 0.8 Å. The significant sequence conservation of CmeR and QacR spans the entire N-terminal region and extends into the N-terminal end of helix \( \alpha 4 \). Despite these structural and sequence similarities, the structure of the DNA-binding domain of CmeR presents some noticeable differences from the rest of the TetR family members. One difference comes from helix \( \alpha 1 \). This first helix consists of 23 residues, which is relatively long compared with all structurally known members of the TetR family. For example, helices \( \alpha 1 \) in QacR, TetR, and EthR are composed of 16, 13, and 17 residues, respectively.

Perhaps, the most striking difference between structures of CmeR and other TetR members in the DNA-binding domain is the lack of the third N-terminal helix in CmeR. Until now, all known TetR family of regulators possess a helix-turn-helix (HTH) DNA-binding motif, which is formed by helices \( \alpha 2 \) and \( \alpha 3 \). Helix \( \alpha 3 \) is named the “recognition helix,” as it has a key role in binding the target DNA. In the case of CmeR, however, only two N-terminal helices, \( \alpha 1 \) and \( \alpha 2 \), are found. According to the sequence alignment, residues 47–53 are supposed to form the recognition helix \( \alpha 3 \). The crystal structure, however, shows that this region forms a random coil. We predicted the secondary structure of CmeR using the programs GOR V\(^25\) and PROF\(^26\). Both predictions describe the overall crystal structure of CmeR quite accurately, and they both exclude the presence of \( \alpha 3 \).

One of the unique features of the CmeR structure is its large center-to-center distance between two N termini of the dimer. As CmeR does not have helix \( \alpha 3 \), we measured this center-to-center distance according to the separation between \( \alpha \) atoms of Tyr51 and Tyr51’,
which was measured 54 Å. The corresponding distances are 39 Å and 35 Å in the apo forms of QacR,\textsuperscript{19} and TetR.\textsuperscript{27} These center-to-center distances increase upon ligand binding. For the ligand-bound dimers of QacR,\textsuperscript{19} TetR\textsuperscript{18} EthR,\textsuperscript{24} and YfiR,\textsuperscript{28} these distances become 41 Å, 38 Å, 52 Å and 54 Å, respectively. Thus, the relatively large center-to-center distance observed with CmeR suggested that CmeR was liganded.

**C-terminal domain**

The C-terminal domain of CmeR consists of eight helices (\(\alpha_4\)–\(\alpha_{10}\)). Except helices 6 and 9, these helices form an anti-parallel, five-helix bundle. In view of the crystal structure, helices 6, 8, 9 and 10 are involved in the formation of the dimer. Dimerization occurs mainly by couplings between pairs of helices (\(\alpha_6\) and \(\alpha_9\), \(\alpha_8\) and \(\alpha_{10}'\), and their identical counter pairs). A surface area of 1950 Å\(^2\) per monomer (probe radius of 1.4 Å) is buried in the contact region of the dimer. The interaction surface is mostly hydrophobic in character. Within 3.5 Å, the close contact pairs in the helices involve I130 and I180', K160 and H193', I114 and Y120', Y153 and I205', L161 and F196', Y116 and Y116', and T167 and P172'. Additional contact interfaces are provided by the loop connecting helices 6 and 7, and the loop region right after helix 10. At the flexible loop between helices \(\alpha_6\) and \(\alpha_7\), Y120 makes contact with I114' in helix \(\alpha_6\)', while residue I205 close to the end of the C-terminus forms hydrophobic contacts with M154' and L202' from the other subunit. The dimer interface is further strengthened by two cross-interface hydrogen bonds formed between backbone atoms (between L202 and I205', and between K113 and V119'), and a water-mediated hydrogen bond (between Y120 and A110'). The overall structure of the C-terminal domain of CmeR is closest to that of QacR among the TetR family members. Superposition of the C-terminal domains of CmeR and QacR suggests that helices 4–6, 7a-b, 8, and 10 of CmeR correspond to helices 4–6, 7, 8, and 9, respectively, of QacR. In QacR, helix \(\alpha_8\) transits directly to the last helix through a nearly 180° turn.\textsuperscript{19} Similar anti-parallel arrangements of the last two C-terminal helices are found in other TetR family members, such as EthR,\textsuperscript{23} YfiR,\textsuperscript{28} and CprB,\textsuperscript{22} and these last two helices are contribute mainly to dimerization. The C-terminal domain of CmeR is distinct, in that helix, \(\alpha_9\), which is between the two anti-parallel helices \(\alpha_8\) and \(\alpha_{10}\), deviates from the direction of \(\alpha_8\) by 40°. Thus, helix \(\alpha_9\) bends toward the next
subunit of the dimer, interacting with α6′ and α7’a to secure interaction between the dimer. The C-terminal domain of CmeR forms a large tunnel-like cavity (Figure 2). This tunnel, formed predominantly by helices 4–9, opens horizontally from the front to the back of each protomer. The length of this tunnel is about 20 Å. Helices α7 and α8 from one subunit, and α9′ from the other subunit of the regulator make the entrance of the tunnel. Residues I130, Q134, F137 and Y139 of α7b; E159, V163 and T167 of α8; and P172′, Y173′, L176′ and I180′ of α9′ are involved in the formation of this entrance.

Surrounding the inner wall of this tunnel are I68, C69, F99, F103, A108, F137 and S138, in which many of these residues are hydrophobic in nature. Helices 4–6, with the side-chains of I68, H72, I102, E107 and F111, contribute to form the end of this hydrophobic tunnel. Similar hydrophobic tunnels have been found in the EthR[23,24] and YfiR28 repressors. In the case of EthR, the tunnel opens vertically to the bottom of the molecule.23,24 For YfiR, however, the long tunnel opens on one end at the subunit interface, and this end of the tunnel is nearly blocked by the second subunit.28

**CmeR was liganded**

The initial solvent-flattened multiple-wavelength anomalous dispersion map showed a positive density, presumably from an unidentified ligand that was purified and crystallized with the protein, inside the hydrophobic tunnel of CmeR. We used the program “putative active sites with spheres” (PASS)29 to search for potential ligand-binding sites in the CmeR structure, in which the top two predicted binding sites were located inside the hydrophobic tunnel. One of these two predicted sites overlaps with the unidentified positive electron density. The simulated annealing omit map shows that the ligand density appears to come from a small molecule.

Gas chromatography coupled with mass spectrometry (GC-MS) suggests that the bound ligand is glycerol (1,2,3-propanetriol), as it was detected as the major component present in the extraction solvent (see Supplementary Data Figure S1). We used solutions containing glycerol to purify and crystallize the protein and it was not surprising that the
identified ligand is glycerol, although the finding was unexpected. The chemical structure of glycerol is compatible with the positive density in the simulated annealing omit map, and it fits unambiguously into the CmeR structure (Figure 3).

Glycerol has not been proved to be a ligand of CmeR or an inducer of the cmeABC operon, the structure obtained probably mimics the ligand-bound form of CmeR. For the glycerol binding, the hydroxyl atom O3 forms hydrogen bonds with S138 and the backbone N atom of Y139 at distances of 3.1 Å and 2.7 Å, respectively. The repressor protein further anchors the bound glycerol through two water-mediated hydrogen bonds, between T167 and hydroxyl atom O2 (through OW1) and between S138 and hydroxyl atom O1 (through OW2) of the bound glycerol. The SG of C166 and NZ of K170 are less than 4 Å away from O2 and O1, respectively, interacting with the bound glycerol and securing the binding.

**Predicting the structure of the DNA-bound form of CmeR**

Although we expect that the DNA-binding mode of CmeR is similar to that of TetR, the overall crystal structure and sequence alignment suggest that the CmeR protein is more similar to the QacR repressor. Thus, a speculative model of DNA-bound CmeR was generated by the alignment of its individual domains with those of the DNA-bound QacR (Figure 4). This model reveals an extensive movement in CmeR that might allow a shift from a ligand-bound form to a DNA-bound form of the repressor, although we cannot exclude the fact that the target DNA itself may also undergo conformational changes allowing binding to CmeR as seen for TetR, QacR and BmrR. On the basis of this DNA-bound model, it is speculated that helix $\alpha_6$ moves toward $\alpha9$ during DNA binding, resulting in a decrease in volume of the ligand-binding site.

**Docking of ligands into the hydrophobic tunnel**

To elucidate different binding modes of CmeR to a variety of ligands, we used the program MEDock to identify potential binding pockets for two bile acids, taurocholate and cholate. We first predicted a glycerol-binding site in CmeR (Figure 5(a)). This predicted site overlaps with the glycerol-binding site identified from the crystal structure, suggesting
MEDock is sufficiently precise for identification of potential protein-binding pockets. When MEDock was used to search for a taurochloate-binding site in CmeR, it was found that the taurocholate molecule was bound inside the tunnel, spanning the two PASS predicted ligand-binding sites. Taurocholate is a 19 Å long ligand, and spans almost the entire length of the ligand-binding tunnel (Figure 5(b)). For the predicted cholate binding in CmeR, cholate binds inside the tunnel, very similar to taurocholate binding (Figure 5(c)).

**Discussion**

The structural similarity of the N-terminal domains of members of the TetR family suggests a similar mode of interaction with target DNAs. CmeR represses the transcription of *cmeABC* by binding directly to the inverting 16 bp IR sequence in the promoter region of the efflux operon. This IR sequence is similar in length to that of the 15 bp *tetO* bound by TetR, but is different from the long 28 bp IR1 recognized by QacR. TetR binds as a single dimer to the *tetO* operator; however, two dimers of QacR interact with one IR1. On the basis of the IR sequence, we reasoned that CmeR might bind its operator as a dimer, similar to the TetR DNA binding.

The separation between two successive major grooves of a 16 bp double helix should be less than 40 Å (the distance between two consecutive major grooves of *B*-DNA is 34 Å). The ligand-bound structure of CmeR indicates that the two DNA recognition regions of the dimer are separated by 54 Å, which is incompatible with the binding of the regulator to its 16 bp operator. Thus, it is possible that a drastic change in conformation of the DNA-binding domain of CmeR might take place during the process of binding target DNA. This change should be greater compared with that of QacR and TetR. In the case of QacR, the change in conformation is accompanied by an increase in the center-to-center distance from 37 Å of the DNA-bound form to 48 Å of the ligand-bound form. For TetR, however, this change is less obvious. The center-to-center distance of the TetR dimer shifts only from 35 Å in the DNA-bound structure to 38 Å in the ligand-bound structure. On the basis of the DNA-bound model of CmeR, it is speculated that this center-to-center distance may decrease to 36 Å upon DNA binding (Figure 4). This change may trigger a coupling movement of helices α6.
and α9, resulting in a decrease in size of the ligand-binding tunnel, which in turn hinders the inducer ligand to enter the ligand-binding site. The consequence is that the inducer ligand has to overcome this steric hindrance in order to bind CmeR. Thus, it is likely that CmeR induction may be governed by steric repulsion that takes place during inducer binding. The crystal structures of both ligand-free and DNA-bound CmeR would be necessary to infer the mechanisms of CmeR induction, and to confirm our speculative models based on the glycerol-bound CmeR structure.

The lack of the recognition helix, α3, in the DNA-binding domain of the CmeR structure is unique among members of the TetR family. Secondary structure prediction using programs GOR V\textsuperscript{25} and PROF\textsuperscript{26} also suggests that this segment (residues 47–53) is likely to form a random coil. An α-helix is stabilized mainly by a favorable enthalpic contribution from the formation of the backbone hydrogen bonds and van der Waals interactions.\textsuperscript{32,33} However, a random coil is mostly favored by conformational entropy that degenerates different conformational states of the coil.\textsuperscript{34} The entropic cost of fixing the backbone dihedral angles in forming an α-helical structure is within 2 kcal/mol at 25 °C.\textsuperscript{33,35,36} We reasoned that in the case of CmeR, the energy difference between α-helical and randomly coil states of the recognition helix are very close, in the range of 1–2 kcal/mol. If this is the case, it is possible that the segment forming α3 is more favorable for formation of the flexible coil state in the absence of target DNA. This segment will transform into an α-helical conformation upon DNA binding due to the release of energy from the formation of hydrogen bonds and contact interactions between the repressor and target DNA. On the basis of a helix propensity scale,\textsuperscript{37} we estimated the amount of energy involved in helix formation of the last five residues in the recognition segment. This estimated energy in CmeR is 3.3 kcal/mol, which is about 1 kcal/mol greater when compared with those of TetR (2.5 kcal/mol), QacR (2.5 kcal/mol), EthR (2.7 kcal/mol), and CprB (1.9 kcal/mol). It seems that in CmeR this segment has less tendency to form α-helix. However, this extra 1 kcal/mol is not excessive and can be compensated easily by the release of energy during repressor–operator interaction.

One striking feature of the CmeR structure is the large ligand-binding tunnel in each monomer. This tunnel, nearly 20 Å in length, is surrounded by mostly hydrophobic residues
of helices 4–9, and occupies a volume of about 1000 Å$^3$. Each hydrophobic tunnel spans horizontally across the C-terminal domain, and can be seen through from the front to the back of the dimer without obstruction. This unique feature, in the TetR family of regulators, highlights the flexibility of the CmeR regulator. Unexpectedly, the crystal structure of CmeR revealed the presence of a glycerol molecule inside this large ligand-binding tunnel. Glycerol binds identically in each subunit, as indicated by the crystallographic 2-fold symmetry of the CmeR dimer (Figure 1(b)). This ligand-binding mode is different from that of QacR, in which one dimer of QacR binds one drug, but similar to that of TetR, which interacts with tetracycline in a 1:1 monomer/drug molar ratio. The volume of the ligand-binding tunnel of CmeR is large enough to accommodate a few of the ligand molecules. Additional water molecules fill the portion of the large tunnel that is unoccupied by ligand. When PASS$^{29}$ was used to search for potential ligand-binding sites in the CmeR structure, the top two predicted sites, which are 8.4 Å apart, were found inside this hydrophobic tunnel. One of these predicted sites corresponds to the glycerol-binding site. The second predicted site, however, is still empty. Thus, CmeR might be able to accommodate a much bigger ligand that spans across these two predicted binding sites. There is also a good chance that CmeR, like QacR, could bind two drug molecules at a time. In any case, the flexibility of the large ligand-binding tunnel suggests that CmeR is a multiple ligand-binding protein.

Although the ligand-binding tunnel is mainly hydrophobic in nature, the electrostatic surface diagram (Figure 2(a)), somewhat surprising, displays a patch of positive surface potential inside the tunnel. This positive potential indicates that CmeR may be more favorable to bind neutral and negatively charged ligands. In fact, many of the CmeR ligands, such as bile acids, are negative in charge. To examine how CmeR binds a variety of ligands, we used MEDock to predict the binding sites of cholate and taurocholate. The docking study suggested that the bound cholate and taurocholate, respectively, occupied both of the PASS predicted sites in the hydrophobic tunnel. These binding modes are similar to that of the dequalinium binding in QacR, in which the bound dequalinium took both the rhodamine 6G and ethidium-binding sites.$^{19}$
CmeR represses the expression of the CmeABC efflux pump that extrudes various bile salts, such as cholate, deoxycholate and taurocholate. It also recognizes commonly used antibiotics, including fluoroquinolones, macrolides, tetracycline and rifampin. Thus, these compounds are the CmeR ligands. It has not been shown that glycerol is a natural ligand of CmeR. The biological effect of CmeR binding by glycerol remains to be determined. Recently, it has been shown using DNA microarray that CmeR may function as a pleiotropic regulator modulating the expression of multiple membrane transporters, including two C4-dicarboxylate transporters DcuA and DcuB (Q.Z., unpublished results). This finding suggests that C4-dicarboxylates, such as malate, fumarate, succinate and aspartate, might be ligands for CmeR. The chemical structures of glycerol (which is bound by CmeR) and C4-dicarboxylates are quite similar, suggesting that CmeR may recognize and respond to C4-dicarboxylate compounds.

Materials and Methods

Purification, crystallization and data collection

Recombinant CmeR, containing a His$_6$ tag at the N terminus, was produced in *Escherichia coli* using the pQE30 vector. The cloning, expression, purification and crystallization procedures have been described.$^{5,9,10,39}$ Diffraction data sets of both the native and SeMet-CmeR crystals were taken at the Advanced Light Source (beamline 8.2.2) at cryogenic temperature (100 K) using an ADSC Quantum 315 CCD-based detector.

Structural determination and refinement

Diffraction data sets were processed with DENZO and scaled with SCALEPACK.$^{40}$ Both native and SeMet crystals took the space group group of $P2_12_12$, with the unit cell parameters given in Table 1. Initial phase calculation was carried out at 2.8 Å resolution using the program BnP$^{41}$ after finding and refinement of all three selenium sites. The electron density map obtained was applied to density modification (DM) using the program RESOLVE.$^{42}$ The auto-interpretation routine program in RESOLVE led to an initial model
containing 78% amino acid residues, 50% of which contained side-chains. The remaining part of the model was constructed manually using the program O. The model, comprising residues 6–207, was then refined against the native data at 2.2 Å using the programs CNS and REFMAC5. Solvent atoms were initially built using the program ARP/warp and later added or removed by manual inspection. The final $R$-factor and $R_{\text{free}}$ (calculated with 5% of the reflections omitted from the refinement) were 21.9% and 24.3%, respectively.

**Modeling of DNA-bound form of CmeR**

The model of the DNA-bound form of CmeR was generated using O. In brief, the N and C-terminal domains of CmeR were separately aligned with the corresponding domains of the DNA-bound QacR (1JT0). The resulting model was then idealized using REFMAC5. The CmeR dimer was generated by applying symmetry operators obtained from the ligand-bound CmeR crystal structure. The recognition helix $\alpha_3$ was also placed accordingly. The final center-to-center distance of the DNA-bound form of CmeR is 36 Å.

**Prediction of ligand-binding sites**

The MEDock web server was used for prediction of the taurocholate (PDB tch) and cholate (PDB chd) bindings in CmeR. A global search strategy that exploits the maximum entropy property of the Gaussian distribution was employed. For the docking protocol, the maximum generation in each run was set to 1000. A grid of 0.375 Å spacing was used for the calculation. Five separate docking calculations were performed for each ligand. Each calculation was performed with a population size of 50, and a probability of 0.05 to invoke local search.

**Identification of fortuitous ligand**

We used GC-MS to identify the nature of the bound ligand in crystals of CmeR. The CmeR crystals were washed extensively with the crystallization buffer and transferred into deionized water. The mixture was incubated at 100 °C for 5 min, and subsequently methanol was added into the mixture to a final concentration of 80% (v/v) to denature the protein and
allow for the extraction of ligand. The GC-MS results suggested that the bound ligand is glycerol (1,2,3-propanetriol) (see Supplementary Data).

**Protein Data Bank accession code**

Coordinates and structural factors for the structure of CmeR have been deposited with the RCSB Protein Data Bank with accession code 2QCO.

**References**


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Figures and Captions

Figure 1. Stereo view of the experimental density map and ribbon diagram of CmeR. (a) Representative section of electron density at the subunit interface. The solvent-flattened electron density (50–2.3 Å) is contoured at the 1σ level and superimposed with the final refined model (yellow, carbon; red, oxygen; blue nitrogen; green, sulfur). (b) Ribbon diagram of the ligand-bound CmeR homodimer generated by crystallographic symmetry. The Figure was prepared using PyMOL [http://www.pymol.sourceforge.net].
Figure 2. Views of the tunnel-like cavity in the ligand-binding domain of CmeR. (a) Electrostatic surface potential of one subunit of CmeR. This view shows the long tunnel spanning through the C-terminal domain of CmeR. Blue (+15 $k_B T$) and red (−15 $k_B T$) indicate the positively and negatively charged areas of the protein, respectively, (b) View of the hydrophobic cavity with residues forming the tunnel. The Figure was prepared using PyMOL [http://www.pymol.sourceforge.net].
Figure 3. Simulated annealing omit map of the glycerol binding pocket. A stereo view of the composed electron density omit map (contoured at the 1.5 σ level) calculated by excluding glycerol from the model. Carbon atoms are colored grey for bound glycerol and light orange for protein residues. Nitrogen, oxygen, and sulfur atoms are colored blue, red, and orange, respectively. The two top binding-site centers predicted from PASS are depicted as pink dotted-spheres. Two water molecules (OW1 and OW2) located at the glycerol-binding site are shown as red balls.
Figure 4. Speculative model of CmeR in its DNA-bound form. The N and C-terminal domains of the ligand-bound dimeric CmeR were individually aligned with those of the DNA-bound QacR (1JT0) to generate the DNA-bound form of CmeR. The two DNA recognition α3 helices in the dimer of CmeR are included in the model.
Figure 5. Binding site prediction for CmeR. (a) CmeR complexed with glycerol. The bound glycerol molecules from the crystal structure and from prediction are colored pink and yellow, respectively. (b) CmeR complexed with taurocholate. The bound taurocholate from prediction is colored orange. (c) CmeR complexed with cholate. The bound cholate from prediction is colored yellow. All predictions were done using MEDock. The two top binding-site centers predicted from PASS are depicted as green dotted-spheres.
CHAPTER 6. Crystallization and preliminary X-ray diffraction analysis of the multidrug efflux transporter NorM from *Neisseria gonorrhoeae*


Chih-Chia Su, Feng Long, Gerry McDermott, William M. Shafer and Edward W. Yu

Abstract

The crystallization and preliminary X-ray data analysis of the NorM multidrug efflux pump produced by *Neisseria gonorrhoeae* are reported. NorM is a cytoplasmic membrane protein that consists of 459 amino-acid residues. It is a member of the recently classified multidrug and toxic compound extrusion (MATE) family of transporters and recognizes a number of cationic toxic compounds such as ethidium bromide, acriflavin, 2-\textit{N}-methyleneellipticinium and ciprofloxacin. Recombinant NorM protein was expressed in *Escherichia coli* and purified by metal-affinity and gel-filtration chromatography. The protein was crystallized using hanging-drop vapor diffusion. X-ray diffraction data were collected from cryocooled crystals at a synchrotron light source. The best crystal diffracted anisotropically to 3.8 Å and diffraction data were complete to 6.5 Å resolution. The space group was determined to be \textit{C}2, with unit-cell parameters \(a = 81.5\), \(b = 164.4\), \(c = 111.5\) Å.
Introduction

Gonorrhea is one of the most common sexually transmitted diseases in the world. It is estimated that 800,000 cases of gonorrhea occur annually in the USA at a cost of $1.1 billion (http://www.thebody.com/content/art6532.html). The disease is caused by the Gram-negative bacterium *Neisseria gonorrhoeae*, which is increasingly resistant to most inexpensive antibiotics, including penicillin, tetracycline, erythromycin and ciprofloxacin. In April 2007, the Centers for Disease Control and Prevention (CDC) officially added gonorrhea to the list of antibiotic-resistant 'superbugs' (http://www.cdc.gov/std/Gonorrhea/STDFact-gonorrhea.htm). Since *N. gonorrhoeae* is a strict human pathogen and can colonize male and female genital mucosal surfaces and other sites, it has developed mechanisms to overcome the host antimicrobial systems that are essential to innate host defense (Shafer *et al.*, 2001). One important mechanism that *N. gonorrhoeae* uses to subvert antimicrobial agents is the expression of multidrug efflux transporters. These transporters recognize and actively export a wide variety of structurally unrelated toxic compounds, including antibacterial peptides, long-chain fatty acids and several clinically useful antibiotics, from the bacterial cell (Lee & Shafer, 1999; Shafer *et al.*, 1998, 2001; Rouquette-Loughlin *et al.*, 2003).

Four efflux transporters have been identified in *N. gonorrhoeae*. One such transporter is the MtrD inner membrane protein (Hagman *et al.*, 1997), which exists as a component of the tripartite resistance nodulation cell division (RND) efflux pump (Tseng *et al.*, 1999). This pump mediates the export of hydrophobic antimicrobial agents including antibiotics, nonionic detergents, certain antibacterial peptides, bile salts and gonadal steroid hormones from the bacterium (Shafer *et al.*, 1998; Delahay *et al.*, 1997; Hagman *et al.*, 1995, 1997). The FarB transporter (Lee & Shafer, 1999), which belongs to the major facilitator (MF) family (Griffith *et al.*, 1992; Marger & Saier, 1993; Pao *et al.*, 1998), recognizes antibacterial long-chain fatty acids and exports them out of the cell. MacB has recently been described (Rouquette-Loughlin *et al.*, 2005) and belongs to the ATP-binding cassette (ABC) transporter family (Higgins, 1992). It is poorly expressed in wild-type gonococci owing to a natural mutation in its promoter, but can recognize and export certain macrolide antibiotics.
Finally, *N. gonorrhoeae* contains the NorM multidrug transporter (Rouquette-Loughlin *et al.*, 2003), which is a member of the most recently classified multidrug and toxic compound extrusion (MATE) family of efflux pumps (Rouquette-Loughlin *et al.*, 2003; Brown *et al.*, 1999; Morita *et al.*, 1998). As a multidrug efflux pump, the gonococcal NorM appears to recognize a number of cationic toxic compounds such as ethidium bromide, acriflavin, 2-N-methylellipticinium and ciprofloxacin (Rouquette-Loughlin *et al.*, 2003). We recently determined that NorM binds a variety of structurally dissimilar agents in the micromolar range and behaves as an Na⁺-dependent transporter (Long *et al.*, 2008). The capacity of NorM to export the antibiotic ciprofloxacin is recognized as being clinically relevant in the development of fluoroquinolone resistance in *N. gonorrhoeae*.

The MATE transporters characteristically possess 12 putative transmembrane domains. To date, the overall properties of the MATE family of proteins have not been completely determined and no structural models are available for this type of multidrug resistance-conferring protein. As an initial step to elucidate the structural basis of multidrug recognition and extrusion by NorM, we here report the crystallization and preliminary X-ray diffraction analysis of the NorM transporter.

**Cloning, expression and purification**

**2.1. Cloning**

Recombinant full-length NorM containing a 6×His tag directly attached to the C-terminal end (C-6×His) was produced in *Escherichia coli* TOP10 cells using the pBAD vector (Invitrogen). The cloning and expression procedures have been described previously (Long *et al.*, 2008). This recombinant C-6×His NorM is fully functional *in vivo* and the purified protein has been demonstrated to bind antimicrobials in a detergent environment with dissociation constants spanning the micromolar range (Long *et al.*, 2008).

To produce recombinant full-length NorM containing a 6×His tag at the N-terminus, the ORF for *norM* from the genomic DNA of *N. gonorrhoeae* strain FA19 was amplified by PCR using the primers 5'-AAACATATGCTGCTCGACCGC-3' and 5'...
AAAGGATCCTCAGACGGCCTTGTGTGATTTGC-3’. The 1380 bp PCR fragment of the *norM* gene with flanking sequences was extracted from the agarose gel using a gel-extraction kit (Qiagen) and then digested with *Nde*I and *Bam*HI (New England Biolabs). The digested products were ligated into the pET15b expression vector (Novagen) to generate a recombinant protein that contains a 6×His tag, a thrombin-cleavage site and a three-residue (GSH) N-terminal spacer attached to the N-terminal end of NorM (N-6×His). The sequence of this N-6×His NorM protein is MGSSHHHHHHHSSGLVPRGSH-NorM. This recombinant plasmid (pET15b*norM*) was transformed into DH5α cells and selected on LB plates containing 100 µg ml⁻¹ ampicillin. The construction was verified by DNA sequencing.

### 2.2. Protein expression

The C-6×His NorM protein was overproduced in *E. coli* TOP10/pBAD*norM* cells as described elsewhere (Long *et al.*, 2008). As these cells cannot be grown in M9 minimal media, recombinant selenomethionyl-NorM (SeMet-NorM) protein was overproduced using the N-6×His construct in *E. coli* B834/pET15*norM* cells. Briefly, a 10 ml overnight culture in Luria-Bertani (LB) broth was transferred into 120 ml LB broth containing 100 µg ml⁻¹ ampicillin. The culture was grown with shaking (210 rev min⁻¹) at 310 K. When the OD₆₀₀ value reached 1.2, cells were harvested by centrifugation at 6000 rev min⁻¹ for 10 min and then washed two times with 20 ml M9 minimal salts solution. The cells were resuspended in 120 ml M9 media and then transferred into 12 l pre-warmed M9 solution containing 100 µg ml⁻¹ ampicillin. The cell culture was incubated at 310 K with shaking. When the OD₆₀₀ reached 0.4, 60 mg l⁻¹ L-selenomethionine was added. The culture was then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) after 15 min. Cells were harvested within 3 h and were frozen and stored at 193 K.

### 2.3. Protein purification

The C-6×His NorM protein was purified using an Ni²⁺-affinity column as described in Long *et al.* (2008), followed by a G-200 sizing column to enhance purity. This step was also essential to exchange *n*-dodecyl-β-D-maltoside (β-DDM) with different detergents for
crystallization attempts. The procedure for detergent exchange using gel-filtration chromatography was as follows: the purified C-6×His NorM protein in buffer containing 20 mM Na HEPES pH 7.5 and 0.1% β-DEEM was concentrated to a volume of 800 µl (∼10 mg ml⁻¹) using a YM-100 concentrator (Millipore, 100 kDa molecular-weight cutoff). The concentrated protein was then loaded onto a Superdex 200 (G-200) 16/60 column (Amersham Pharmacia Biotech) pre-equilibrated with buffer containing 20 mM Na HEPES pH 7.5 and the relevant detergent at the described concentration. The volume and length of the G-200 sizing column were 120 ml and 60 cm, respectively. A flow rate of 0.5 ml min⁻¹ was used and 2 ml fractions were collected and analyzed by 10% SDS-PAGE.

The purification procedures for the N-6×His SeMet-NorM protein were similar to those used for C-6×His NorM. In brief, the N-6×His SeMet protein was purified using an Ni²⁺-affinity column as described in Long et al. (2008). The purified protein was extensively dialyzed against buffer containing 20 mM Na HEPES pH 7.5 and 0.1% β-DEEM, concentrated to 2 mg ml⁻¹ and then incubated for 24 h at 298 K in the presence of one unit of thrombin per 2 mg protein to cleave the hexahistidine tag. After thrombin cleavage, the newly formed SeMet-NorM protein, which contained a three-residue spacer, GSH, directly attached to the N-terminus (GSH-SeMet-NorM), was loaded onto a G-200 sizing column pre-equilibrated with buffer containing 20 mM Na HEPES pH 7.5 and 0.1% β-DEEM for further purification. The purity of the purified GSH-SeMet-NorM protein was judged using 10% SDS-PAGE stained with Coomassie Brilliant Blue. NorM is a 459-amino-acid protein that contains 19 methionines. Replacement of these methionine sulfurs with selenums in the GSH-SeMet-NorM protein was confirmed by MALDI time-of-flight mass spectrometry.

Both the C-6×His NorM and GSH-SeMet-NorM proteins were concentrated to 20 mg ml⁻¹ in solution containing 20 mM Na HEPES pH 7.5 and 0.1% β-DEEM. Typical starting and ending volumes were 10 ml and 300 µl, respectively. To avoid concentrating β-DEEM in the protein solution, a YM-100 Centriprep concentrator (Millipore, 100 kDa molecular-weight cutoff) was used for protein concentration. The 100 kDa molecular-weight cutoff concentrators have been shown to be efficient enough to avoid concentrating the β-DEEM micelles (Urbani & Warne, 2005).
3. Crystallization

3.1. Detergents

The full-length C-6×His NorM protein containing six histidines at the C-terminus was crystallized in 24-well plates using the hanging-drop vapor-diffusion method. Initial crystallization trials using commercially available screening kits, including Hampton Crystal Screens I, II and Lite, PEG/Ion and MembFac, failed. Accordingly, we performed crystallization screening by mixing different polyethylene glycols [PEGs; ranging from PEG 200 to PEG 20 000 and PEG 5500 MME (monomethyl ether) to PEG 5500 MME] with different buffers (between pH 3.5 and pH 9.5), salts, additives and detergents. The experiments were performed at 277 or 298 K.

Initially, five different detergents, α-DDM, n-dodecyl-α-D-maltoside (α-DDM), polyoxyethylene(8)dodecyl ether (C\textsubscript{12}E\textsubscript{8}), n-undecyl-α-D-maltoside (UDM), n-tetradecyl-β-D-maltoside (TDM) and n-octyl-β-D-glucoside (OG), were used as primary detergents in our crystallization trials. The experiments suggested that protein solution containing 0.1% α-DDM or 0.8% OG resulted in the formation of protein crystals. Crystals grown in the presence of α-DDM diffracted to 8 Å; however, those grown in the presence of OG did not diffract X-rays. Thus, α-DDM was chosen as the primary detergent for further crystallization trials.

We attempted to improve the quality of the crystals by searching for a suitable secondary detergent using Detergent Screens 1, 2 and 3 (Hampton Research). After the initial screen, eight different secondary detergents, C\textsubscript{12}E\textsubscript{8}, UDM, polyoxyethylene(9)dodecyl ether (C\textsubscript{12}E\textsubscript{9}), 6-cyclohexyl-1-hexyl-β-D-maltoside (Cymal-6), n-decyl-β-D-maltoside (DM), n-dodecylphosphocholine (Fos-choline-12), n-nonyl-β-D-glucoside (NG) and 3-(3-butyl-3-phenylheptanamido)-N,N-dimethylpropan-1-amine oxide (TRIPAO), were chosen for further trials. However, it appeared that the addition of these secondary detergents did not improve the diffraction limit of the crystals.
3.2. Removal of hexahistidine tags

We then created a thrombin-cleavage site at position 454 of the protein in order to remove the hexahistidine tag at the C-terminus of C-6×His NorM. After removing the 6×His tag, this NorM protein (NorM454, amino acids 1-454) was purified in β-DDM and subjected to a broad screen with various PEGs, salts, buffers, additives and detergents as described above. Unfortunately, crystals of the NorM454 protein diffracted X-rays in a similar manner to those of C-6×His NorM and did not appear to enhance the resolution limit.

We also attempted to improve the crystal quality by crystallizing N-6×His NorM. After the removal of the 6×His tag using thrombin, this NorM protein, GSH-NorM, containing a three-residue spacer (GSH) at the N-terminal end, was screened with various PEGs, salts, buffers, additives and detergents as above. However, the best crystal obtained was no better than that obtained for C-6×His NorM. We thus focused our subsequent crystallization efforts on the C-6×His NorM protein.

3.3. Crystallization conditions

For hanging-drop vapor diffusion, a 2 µl drop consisting of 1 µl protein solution (20 mg ml⁻¹ NorM in 20 mM Na HEPES pH 7.5 and 0.1% β-DDM) and 1 µl reservoir solution was equilibrated against 500 µl reservoir solution. The initial crystals of NorM were grown in 15-18% PEG 1000 and 20-100 mM Na HEPES pH 7.5. After optimization, the best C-6×His NorM crystals were obtained at 298 K from well solution containing 16% PEG 1000, 40 mM Na HEPES buffer pH 7.5 and 4% glycerol. Crystals appeared within one week and typically had dimensions of about 200 × 200 × 200 µm. Fig. 1 illustrates a typical native crystal of C-6×His NorM. Cryoprotection was achieved by raising the PEG 1000 concentration stepwise to 26% in 5% increments. Crystals of the GSH-SeMet-NorM protein (after removal of the 6×His tag at the N-terminus) were grown under the same conditions.
4. Data collection and processing

For data collection, a single native crystal of C-6×His NorM was flash-cooled in a cryoprotectant solution containing 26% PEG 1000, 40 mM Na HEPES buffer pH 7.5 and 4% glycerol at 100 K. The best crystal diffracted anisotropically to a resolution of 3.8 Å. Fig. 2 depicts one of the diffraction images of the native NorM crystal. Single-wavelength anomalous diffraction data were collected at the peak wavelength from a single GSH-SeMet-NorM crystal. The best native C-6×His NorM and GSH-SeMet-NorM diffraction data were obtained to resolutions of 6.5 and 7.2 Å, respectively (Table 1).

Diffraction data sets were obtained from the native C-6×His NorM crystals at the Advanced Photon Source (APS, beamline 24IDC) at cryogenic temperature (100 K) using an ADSC Quantum 315 CCD-based detector. The beam size was 50 × 20 µm. Data for the GSH-SeMet-NorM crystals were obtained at the Advanced Light Source (ALS, beamline 8.2.2) at cryogenic temperature (100 K) using an ADSC Quantum 315 CCD-based detector. The beam size was 140 × 150 µm. Diffraction data sets were processed with DENZO and scaled with SCALEPACK (Otwinowski & Minor, 1997). The native C-6×His NorM crystal belonged to space group C2, with unit-cell parameters \( a = 81.5, b = 164.4, c = 111.5 \) Å. The GSH-SeMet-NorM crystal belonged to the same space group with very similar unit-cell parameters (Table 1). Based on the molecular weight of the C-6×His NorM protein (50.6 kDa, including the 6×His tag at the C-terminus) and the volume of the asymmetric unit, the Matthews parameters (Matthews, 1968, 1977) for one and two molecules of NorM in the asymmetric unit were found to be 7.0 and 3.5 Å³ Da⁻¹, respectively. This suggests the presence of one or two NorM molecules per asymmetric unit, with solvent contents of 82.2% or 64.4%, respectively.

Acknowledgements

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**Figures and Captions**

**Figure 1**
*N. gonorrhoeae* C-6×His NorM crystal. The dimensions of the crystal are approximately 200 × 200 × 200 µm.
Figure 2
X-ray diffraction pattern of the native C-6×His NorM crystal. The crystal diffracted anisotropically to a resolution beyond 3.8 Å.
Table 1

Data collection and crystallographic analysis of NorM

Values in parentheses are for the highest resolution shell.

<table>
<thead>
<tr>
<th></th>
<th>Native C-6xHis NorM</th>
<th>GSH-SeMet-NorM (peak)</th>
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CHAPTER 7. Crystal structure of the membrane fusion protein CusB from *Escherichia coli*

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Abstract

Gram-negative bacteria, such as *Escherichia coli*, frequently utilize tripartite efflux complexes belonging to the resistance-nodulation-division family to expel diverse toxic compounds from the cell. These systems contain a periplasmic membrane fusion protein that is critical for substrate transport. We here present the x-ray structures of the CusB membrane fusion protein from the copper/silver efflux system of *E. coli*. This is the first structure of any membrane fusion proteins associated with heavy-metal efflux transporters. CusB bridges the inner membrane efflux pump CusA and outer membrane channel CusC to mediate resistance to Cu\(^{+}\) and Ag\(^{+}\) ions. Two distinct structures of the elongated molecules of CusB were found in the asymmetric unit of a single crystal, which suggests the flexible nature of this protein. Each protomer of CusB can be divided into four different domains, whereby the first three domains are mostly β-strands and the last domain adopts an entirely helical architecture. Unlike other known structures of membrane fusion proteins, the α-helical domain of CusB is folded into a three-helix bundle. This three-helix bundle presumably interacts with the periplasmic domain of CusC. The N and C-termini of CusB form the first β-strand domain, which is thought to interact with the periplasmic domain of the CusA efflux pump. These findings reveal novel structural features of a membrane fusion protein in the resistance-nodulation-division efflux system, and provide new insight into the mechanisms of heavy-metal extrusion by CusB.
Introduction

Silver is a heavy metal with relatively high toxicity to prokaryotes. Ionic silver exhibits antimicrobial activity against a broad range of microorganisms, and is used widely as an effective antimicrobial agent to combat pathogens. Copper, although required in trace amounts for bacterial growth, is highly toxic even at low concentrations. Thus, both silver and copper are well-known bactericides, and their biocidal effects have been used for centuries. It has been shown that silver and copper ions effectively eliminate Legionella in drinking water pipelines. Silver and copper ions are capable of penetrating biofilms that build up in hospital plumbing, destroying entrenched Legionella and other pathogenic organisms. In addition, silver cations are commonly employed in treating patients with burns, wounds, eye infections and ulcers. To date, the antimicrobial uses of ionic copper have been expanded to include fungicides, antifouling paints, antimicrobial medicines and antiseptics. Because of the widespread use of silver and copper as antimicrobial agents, the presence of silver and copper resistant bacterial strains appear to be on the rise.

Bacteria, such as Escherichia coli, have developed various mechanisms to overcome toxic environments that are unfavorable to their survival. One important strategy that bacteria use to subvert toxic compounds, including toxic metal ions such as Ag\(^+\) and Cu\(^+\), is the expression of membrane efflux transporters that recognize and actively export these compounds out of bacterial cells, thereby allowing them to survive in extremely toxic conditions. In Gram-negative bacteria, efflux systems of the resistance-nodulation-division (RND) family play major roles in the intrinsic and acquired tolerance of antibiotics and toxic compounds. As a Gram-negative bacterium, E. coli contains seven different RND efflux transporters. Six of these transporters, including AcrB, AcrD, AcrF, MdtB, MdtC and YhiV, are multidrug efflux pumps. They belong to the hydrophobic and amphiphilic efflux RND (HAE-RND) protein family. E. coli consists of only one heavy-metal efflux RND (HME-RND) transporter, CusA, which specifically recognizes and confers resistance to Ag(I) and Cu(I) ions.

Typically, an RND transporter works in conjunction with a periplasmic component, belonging to the membrane fusion protein (MFP) family, and an outer membrane channel to form a functional protein complex. The resulting tripartite efflux system spans the inner
and outer membranes of Gram-negative bacterium to export substrates directly out of the cell.\textsuperscript{16} For the CusA inner membrane transporter, it interacts with the periplasmic membrane fusion protein CusB and the outer membrane channel CusC to form the CusABC tripartite efflux complex.\textsuperscript{12,13} Heavy-metal efflux by CusABC is driven by proton import. This process is catalyzed through the inner membrane transporter CusA.

Among all known RND family of transporters, the \textit{E. coli} AcrB\textsuperscript{17-20} and \textit{Pseudomonas aeruginosa} MexB\textsuperscript{21} HAE-RND pumps are the only two membrane proteins that have been crystallized. These proteins span the entire width of the inner membrane and protrude approximately 70-Å into the periplasm. The crystal structures of the outer membrane channels, \textit{E. coli} TolC and \textit{P. aeruginosa} OprM, have also been determined.\textsuperscript{22,23} TolC is anchored in the outer membrane and forms a 100-Å-long periplasmic \(\alpha\)-helical tunnel.\textsuperscript{22} The \textit{P. aeruginosa} OprM channel possesses a similar elongated \(\alpha\)-helical tunnel that projects into the periplasmic space.\textsuperscript{23} Recently, two structures of the periplasmic membrane fusion proteins, \textit{E. coli} AcrA\textsuperscript{24} and \textit{P. aeruginosa} MexA,\textsuperscript{25-27} associated with the HAE-RND transporters have been solved. The structures suggest that these two periplasmic proteins are folded into elongated secondary structures that consist of \(\sim\)47-Å-long \(\alpha\)-hairpin domain, presumably interacting with the \(\alpha\)-helical tunnels of their corresponding outer membrane channels. Further, the N and C-terminal ends of these membrane fusion proteins are thought to contact their respective inner membrane transporters, creating a functional complex that spans both membranes.

Currently, no structural information is available for any components of the HME-RND tripartite efflux complex. Presumably, the three components of the HME-RND system form a tripartite complex that resembles the AcrAB-TolC complex. Different from the HAE-RND family, members of the HME-RND family are highly substrate specific, with the ability to differentiate between monovalent and divalent ions. As an initial step to examine the mechanisms used by the CusABC efflux system to facilitate Ag(I) and Cu(I) ions recognition and extrusion, we here describe the crystal structure, at 3.4 Å resolution, of the periplasmic membrane fusion protein CusB.
Results

Overall structure of CusB

We cloned, expressed and purified the full-length CusB protein containing a 6xHis tag at the C-terminus. We obtained crystals of the *E. coli* CusB efflux protein in detergent following an extensive screening for crystallization conditions. We then used the multiple-wavelength anomalous dispersion method to solve the selenomethionyl-substituted (SeMet) CusB crystal structure. The resulting experimental electron density maps shown in Figure 1 revealed that the asymmetric unit of the CusB crystal consists of two protomers (labeled A and B). These two molecules are related to each other by an approximately twofold symmetry. The native crystal structure of CusB was then determined to a resolution of 3.4 Å (Table 1), revealing that the A molecule of CusB is folded into an elongated polypeptide of ~121 Å long and ~37 Å wide; whereas the dimensions of the B molecule are ~116 Å long and ~40 Å wide. The mature protein of CusB consists of 379 amino acids (residues 29 through 407). Currently, 78.1% of the residues are included in our final model.

Intriguingly, the crystal structure suggested that each elongated molecule of CusB can be divided into four different domains (Figure 2). The first three domains of the protein are mostly β-strands. However, the fourth domain forms an all α-helical domain, which is folded into a three-helix bundle secondary structure. Alignment of amino acid sequences indicates that CusB shares 13% identity and 52% similarity to that of MexA. The alignment also shows an overall identity and homology of 16% and 54% to AcrA, respectively. Because of the relatively low sequence identity, it is not surprising that the crystal structure of CusB is quite different from the known structures of MexA\textsuperscript{25-27} and AcrA.\textsuperscript{24}

The β-strand domains

As mentioned earlier, each CusB molecule consists of three different β-domains. The first β-domain (Domain 1) is formed by the N and C-terminal ends of the polypeptide (residues 89-102 and 324-385). Presumably, this domain is located directly above the outer-leaflet of the inner membrane and interacts with the CusA efflux pump. Overall, Domain 1 in molecule A is a β-barrel domain. It is composed of six β-strands, with the N-terminal end forming one of the β-strands while the C-terminus of the protein constitutes the remaining five strands (Figure 2).
Domain 1 in molecule B of CusB exhibits an almost identical structure, which is also folded into a six β-barrel domain.

The second β-domain (Domain 2) of CusB is formed by residues 105-115 and 243-320. In molecule A, this domain consists of six β-strands and one short α-helix. Again, the N-terminal residues form one of the β-strands that is incorporated into this domain. The C-terminal residues contribute a β-strand, an α-helix and four anti-parallel β-sheets. In molecule B, this domain differs by assembling into six β-strands and two short α-helices. In an asymmetric unit of the crystal, Domains 1 and 2 of molecule A interact closely with Domains 2 and 1 of molecule B, respectively (Figure 1a).

Domain 3 is another globular β-domain adjacent to the second domain of molecule A. This domain consists of residues 121-154 and 207-239, with a majority of these residues folding into eight β-strands. Similar to that of molecule A, the corresponding domain in molecule B is also an eight β-barrel structure.

The α-helical domain

Perhaps the most interesting motif appears to be in the fourth domain (Domain 4) of CusB. This region forms an all-helical domain. In molecule A, this α-domain comprises residues 156-205. Surprisingly, this domain is folded into an anti-parallel, three-helix bundle. This structural feature, not found in other known protein structures in the MFP family, highlights the uniqueness of the CusB protein. The helix bundle creates an ~27 Å long helix-turn-helix-turn-helix secondary structure, making it at least 20 Å shorter than the two-helical hairpin domains of MexA25-27 and AcrA.24 Domain 4 of molecule B exhibits a similar three-helix bundle motif when compared to the secondary structure of molecule A. To date, CusB is the only periplasmic protein in the MFP family that possesses this three-helical domain instead of a two-helical hairpin motif. The overall structure of CusB is quite distinct from the known structures of other membrane fusion proteins.

It is interesting to note that both crystals of CusB and MexA27 contain two copies of the molecules, each of which consists of four different domains. We superimposed these protein structures and observed that the pair containing molecule A of CusB and the “unrotated” molecule of MexA displays the closest topological similarity (Figure 3). A pairwise alignment
of these two structures is difficult because their detailed secondary structures are quite different. We tried to superimpose the α-domains (Domain 4 of CusB and the α-hairpin domain of MexA) of these two structures together and found that these two α-domains cannot be aligned. However, their individual β-domains can be superimposed separately. For example, superposition of Domain 1 of CusB with the membrane proximal domain of MexA gives an overall rmsd of 2.4 Å calculated over the Cα atoms. Domain 2 of CusB and the β-barrel domain of MexA can also be superimposed, resulting in an overall rmsd of 2.0 Å. Likewise, Domain 3 of CusB can be paired up with the lipoyl domain of MexA, and this superimposition results in an overall rmsd of 1.8 Å.

**Conformational flexibility of CusB**

Two distinct conformations of CusB were captured in the single crystal, suggesting that the membrane fusion protein is quite flexible in nature. A comparison of the A and B molecules of CusB indicates that these two molecules are quite different, presumably representing two transient states of the membrane fusion protein. However, the two conformations are related, whereby a small hinge motion is attributed to the differences. Superimposition of these two molecules gives an overall rmsd of 2.6 Å calculated over the Cα atoms. Comparison of these two structures reveals that molecule A adopts a more open conformation, while molecule B exhibits a relatively compact form of the structure (Figure 4). Thus, these two molecules may correspond to the open and closed states of the membrane fusion protein.

It appears that Domains 1 + 2 of molecules A and B can easily be superimposed with high structural similarity, giving an overall rmsd of 0.8 Å (168 Cα atoms). Superposition of Domains 3 + 4 alone of these two molecules can also be calculated, showing an overall rmsd of 0.8 Å (118 Cα atoms). Using Domain 1 + 2 as references, the α-helical domains of molecules A and B are found to differ by ~21° overall (Figure 4a). When Domains 3 + 4 are superimposed, the orientation of the β-barrels of Domain 1 in molecules A and B display an overall shift of ~23° (Figure 4b). Taken together, these superimpositions suggest that the linker region, which is composed of two loops (residues 116-120 and 240-242) between Domains 2 and 3, forms a flexible hinge in the membrane fusion protein. This hinge region effectively permits the protein to shift from one conformation to another simply by performing a rigid-
body movement at Domains 1 + 2 with respect to Domains 3 + 4. The two structures of CusB found in a single crystal indeed underscore the conformational flexibility of this membrane fusion protein.

**Discussion**

In this study, we presented the crystal structure of the membrane fusion protein CusB, an essential component in the CusABC efflux system which extrudes silver and copper ions from *E. coli*. This is the first structure of any membrane fusion protein that is associated with an HME-RND-type transporter. Currently, CusB and MexA\(^{27}\) exhibit the most complete three-dimensional structures among those resolved for membrane fusion proteins, including AcrA\(^{24}\) and MacA\(^{28}\). The overall structures of MexA, AcrA and MacA are very similar to each other. For example, a superposition of 183 C\(^\alpha\) atoms of AcrA with their corresponding residues in the MexA structure gives an overall rmsd of 0.8 Å\(^{24}\). CusB, however, is folded into a very unique secondary structure when compared with the current crystal structures of other membrane fusion proteins. Like MexA, the structure of CusB revealed that this membrane fusion protein consists of four major domains, including three β-strand domains and one α-helical domain. Strikingly, the α-helical domain of CusB features a three-helix bundle which contrasts other structures of membrane fusion proteins. This structural feature, not found in other members of the MFP family, may underscore the unique functionality of CusB. Thus, CusB may utilize a different mechanism to facilitate metal ion transport in the RND family. The distinct secondary structure of CusB may also imply that its tripartite partners, the inner membrane transporter CusA and the outer membrane channel CusC, may also possess unique secondary structural features that distinguish them from the existing structures of their homologous proteins. Exactly how these individual heavy-metal efflux components assemble into a functional complex must await the elucidation of the CusA and CusC structures.

The crystal structure of CusB demonstrated that this protein exists in two distinct conformations, one of which presents a more open form while the other exhibits a more compact structure. In the crystal lattice, these two molecules (molecules A and B) interact with one another to form a dimer. It should be noted that this dimeric arrangement might not be biologically relevant because, similar to the other isolated membrane fusion proteins associated with the HAE-RND family, *in vitro* study indicated that CusB exists a monomer in solution\(^{29}\).
Thus, the functional form of CusB in solution is most likely to be monomeric. The two distinct conformations of CusB, however, may represent two different transient states of the protein, i.e. the apo and metal-bound forms. Analytical gel filtration experiments suggested that the conformation of CusB shifts to a more compact state upon binding Ag(I). Thus, it is likely that the compact structure, represented by molecule B of CusB, may mimic the metal-bound form of the protein.

The fact that two copies of CusB have been found in a single crystal highlights the conformational flexibility of this membrane fusion protein. The flexible nature of these membrane fusion proteins has also been observed with MexA, AcrA and MacA. There is a good chance that conformational flexibility is a common feature among members of the MFP family. Indeed, four different conformations of AcrA have been identified within a single crystal.

Based on experimental results from extended x-ray absorption fine structure (EXAFS) and site-specific mutagenesis, it has been proposed that residues M49, M64 and M66 of CusB form a three-coordinate metal-binding site for Cu\(^+\) and Ag\(^+\). These residues are located at the N-terminal end of CusB, a region that is intrinsically disordered and cannot be identified in the electron density maps of our crystals. Thus, these residues were excluded in our model. Potentially, these methionine residues could form an ideal binding site for Cu(I)/Ag(I). According to the crystal structures of CusB, this proposed Cu(I)/Ag(I) binding site may be located right above the inner membrane, adjacent to the periplasmic domain of the CusA efflux pump. It is possible that this proposed metal binding site might directly interact with this membrane protein. If this is the case, CusB may capture the metal that is released from CusA through this proposed metal binding site. In addition, if the \(\alpha\)-helices at Domain 4 of CusB interact with the outer membrane channel CusC, this implies that CusB may be involved in delivering the bound metal ions to CusC and eventually exporting the metal ions from the cell. It has also been proposed that CusB directly interacts with the chaperone CusF, which captures the metal ions in the periplasm. There is a chance that the metal ions could be transferred from this chaperone protein to CusB for export. Further experimental evidence is needed to understand the detailed mechanisms of metal ion extrusion in this system.
The mature protein of CusB consists of nine methionine residues. Four of these methionines are located at one end of the disordered region formed by the N and C-termini of the protein. Three of these four methionines, M49, M64 and M66, have been proposed to form a three-methionine metal binding site. Surprisingly, the remaining five methionine residues do not pair up with each other but distribute through the entire length of the protomer. So far, all available structures of copper tolerance proteins, including CusF, CueR and Atx1, indicate that these proteins carry their Ag(I) or Cu(I) cargo in either two-methionine or two-cysteine binding pockets. The bound monovalent metal ions are usually further anchored by histidine and/or tryptophan side chains to secure the binding. Thus, it is uncertain if these five separate methionines could accommodate the binding of Ag(I) and Cu(I). To elucidate if the CusB structure potentially forms ligand-binding sites, we used the program “putative active sites with spheres” (PASS) to search for these sites. It turned out PASS suggests that the top predicted site is located in the vicinity of the hinge region between Domains 2 and 3. Surrounding this potential binding site are residues K143, M241 and K308 (Figure 5). Although, lysine has been reported to interact with Cu$^+$ by capturing and releasing the bound metal ion in the yeast metallochaperone Atx1, this site only contains one methionine. It is not certain if this predicted site could bind metal ions.

There is evidence that members of the MFP family play a functional role in the efflux of substrates. It has been found that the MFP EmrA is able to directly bind different transported drugs. Recently, the CusB protein has also been shown to interact with Ag(I). Thus, in addition to their role as adaptors to bridge the inner and outer membrane efflux components, these membrane fusion proteins may participate in recognizing and extruding their substrates. Exactly how CusB binds heavy metals is not clear. The crystal structures of CusB-Ag(I) and CusB-Cu(I) complexes would be necessary to understand how this membrane fusion protein interacts with metal ions.
Materials and Methods

Cloning, expression and purification

For cloning *cusB*, the ORF of *cusB* from *E. coli* K12 chromosomal DNA was amplified by PCR using the primers 5'-AAACCATGGGCAAAAAAATCGCGCTTTATTATCGGC-3' and 5'-AAAGGATCCTCAATGGTGATGGTGATGATGATGCGCATGGGTAGCACTTTTCAG-3' to generate a product that would encode a CusB recombinant protein with a 6xHis tag at the C-terminus. The corresponding 1,224 bp PCR fragment was extracted from the agarose gel, digested with *Nco*I and *Bam*HI (New England Biolabs), and cloned into the pET15b to form the expression vector, pET15bΩ*cusB*. The recombinant plasmids were transformed into DH5α cells and selected on LB plates containing 100 µg/ml ampicillin. The construction was verified by DNA sequencing.

The full-length CusB membrane fusion protein containing a 6xHis tag at the C-terminus was overproduced in *E. coli* BL21(DE3) cells possessing pET15bΩ*cusB*. Briefly, cells were grown in 12 L of Luria Bertani (LB) medium containing 100 µg/ml ampicillin at 37°C. The culture was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at OD<sub>600</sub> value of approximately 0.5. Cells were harvested within 4 h of induction. The collected bacteria were resuspended in ice-cold buffer containing 20 mM Na-HEPES (pH 7.2) and 100 mM NaCl. The cells were then lysed in a French pressure cell. Cell debris was removed by centrifugation for 45 min at 4°C and 20,000 rev/min. The crude lysate was collected and 0.5% *n*-dodecyl-β-D-maltoside (DDM) was added into the protein solution. The protein solution was then purified with Ni<sup>2+</sup>-affinity and G-200 sizing columns. The purity of the protein (> 95%) was judged using 10% SDS-PAGE stained with Coomassie Brilliant Blue. The N-terminal sequence of the CusB protein was confirmed by sequencing. The purified CusB protein was then concentrated to 20 mg/ml in a buffer containing 20 mM Na-HEPES (pH 7.5) and 0.04% DDM for crystallization.

For 6xHis SeMet-CusB protein expression, a 10 ml LB broth overnight culture containing *E. coli* B834/ pET15bΩ*cusB* cells was transferred into 120 ml of LB broth containing 100 µg/ml ampicillin and grown at 37°C. When the OD<sub>600</sub> value reached 1.2, cells
were harvested by centrifugation at 6000 rev/min for 10 min, and then washed two times with 20 ml of M9 minimal salts solution. The cells were re-suspended in 120 ml of M9 media and then transferred into a 12 L pre-warmed M9 solution containing 100 µg/ml ampicillin. The cell culture was incubated at 37°C with shaking. When the OD$_{600}$ reached 0.4, 60 mg/l of L-selenomethionine were added. The culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after 15 min. Cells were then harvested within 4 h after induction. The procedures for purifying the 6xHis SeMet-CusB were identical to those of the native protein.

Crystallization, data collection, structural determination and refinement

Crystals of the 6xHis CusB were obtained using hanging-drop vapor diffusion. The CusB crystals were grown at room temperature in 24-well plates with the following procedures. A 2 µl protein solution containing 20 mg/ml CusB protein in 20 mM Na-HEPES (pH 7.5) and 0.04% (w/v) DDM was mixed with a 2 µl of reservoir solution containing 15% PEG 1000, 0.1 M Na-citrate (pH 5.6), 0.36 M Na-citrate, 5% isopropanol and 5% glycerol. The resultant mixture was equilibrated against 500 µl of the reservoir solution. The crystallization conditions for SeMet-CusB were the same as those for the native CusB protein. Crystals of CusB grew to a full size in the drops within a month. Typically, the dimensions of the crystals were 0.4 mm x 0.4 mm x 0.1 mm. Cryoprotection was achieved by raising the PEG 1000 concentration stepwise to 30% with a 5% increment in each step.

All diffraction data sets were collected at 100 K in the Advanced Photon Source (beamline 24-ID-C) using an ADSC Quantum 315 CCD-based detector. Diffraction data were processed with DENZO and scaled with SCALEPACK.$^{36}$ Both the native and SeMet crystals took the space group of $I2_22$, with unit cell parameters very similar to each other (Table 1). The structure of CusB was determined by the multiple-wavelength anomalous dispersion (MAD) method. Phase refinement was carried out using the program AutoSHARP.$^{37}$ The electron density map was then subjected to density modification using the program RESOLVE.$^{38}$ After tracing the initial model manually in the program Coot,$^{39}$ the model was then refined against the native data at 3.4 Å resolution using CNS$^{40}$ and PHENIX.$^{41}$

Accession Numbers
Coordinates and structural factors have been deposited in the Protein Data Bank with accession number 3H9I.

Acknowledgments

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References


Legends of Figures

Figure 1a
Figure 1b

Figure 1. Stereo view of the experimental electron density map at a resolution of 3.8 Å. (a) The electron density map contoured at 1.2 $\sigma$ is in gray. The C$^\alpha$ traces of molecules A and B of CusB are in orange and green, respectively. (b) Representative section of the electron density in the second domain of CusB. The electron density (colored blue) is contoured at the 1.2 $\sigma$ level and superimposed with the final refined model (orange, carbon; red, oxygen; blue, nitrogen).
Figure 2. Crystal structure of the CusB membrane fusion protein. The structure can be divided into four distinct domains. Domain 1 is formed by the N and C-termini and is located above the inner membrane. The loops between Domains 2 and 3 appear to form an effective hinge to allow the molecule to shift from an open conformation to a more compact structure. Domain 4 is folded into an anti-parallel, three-helix bundle, which is thought to be located near the outer membrane.
Figure 3. Structural comparison of the membrane fusion proteins. (a) Superimposition of the crystal structures of CusB (orange) and MexA (purple). (b) Superimposition of Domain 1 of CusB (orange) with the membrane proximal domain of MexA (purple). (c) Superimposition of Domain 2 of CusB (orange) with the β-barrel domain of MexA (purple). (d) Superimposition of Domain 3 of CusB (orange) with the lipoyl domain of MexA (purple).
Figure 4. Comparison of the two conformations of CusB observed in the crystal. (a) Superposition of Domains 1 + 2 of molecule A onto Domains 1 + 2 of molecule B, displaying an ~21° overall shift of the three-helix bundle of Domain 4. (b) Superposition of Domains 3 + 4 of molecule A onto Domains 3 + 4 of molecule B, displaying an overall shift of the β-strands of Domain 1 by ~23°.
Figure 5. Binding site prediction for CusB. This predicted binding site is located at the hinge region between Domains 2 and 3 of CusB. Residues K143, M241 and K308 are shown as stick models (yellow, carbon; blue, nitrogen; orange, sulfur).
Table 1. Data collection, phasing and structural refinement statistics.

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| Phasing                  |        |              |            |                   |
| Phasing power            |        | 1.93         |            |                   |
| R_{cullis}               | 0.89   |              |            |                   |
| Figure of merit (acentric/centric) | 0.40/0.26 |              |            |                   |
| Figure of merit after density modification | 0.66/0.62 |              |            |                   |

| Refinement               | CusB   |              |            |                   |
| Resolution (Å)           | 50-3.40|              |            |                   |
| R_{work} (%)             | 26.5   |              |            |                   |
| R_{free} (%)             | 31.7   |              |            |                   |
| Protein residues built   | 592    |              |            |                   |
| rms deviation from ideal |        |              |            |                   |
| Bond lengths (Å)         | 0.008  |              |            |                   |
| Bond angles (°)          | 1.6    |              |            |                   |
CHAPTER 8. General Conclusion and Future Direction

The transport cycle of multidrug efflux pumps must involve various conformational states, including substrate binding and translocation. We have detected the binding affinity of different substrates to the AcrB RND-type transporter. We have also determined structures of different conformational states of AcrB using x-ray crystallography. The key question in these efflux systems is the pathway for substrate translocation and it remains unknown. Combining with site-specific mutagenesis and single molecule technique, we should be able to develop a three-color FRET system and observe the phenomenon of substrate translocation. In the future, we will perform research work in this direction. Using single molecule technique, we should be able to observe a sequential change in FRET signal during drug export. Thus, this approach should allow us to differentiate different stages of the transport cycle.

The crystal structures of different membrane fusion proteins, including CusB, MexA and AcrA, indicate that these proteins are flexible in nature. The role of these flexible proteins and how they interact with their substrates is still not clear. Recent advances in NMR spectroscopy have led us to measure heteronuclear multi-dimensional NMR signal of macromolecules inside living cells. This method, so-called in-cell NMR, has allowed in vivo observations of the structures and dynamics of biomolecules. It is thought that this method is suitable for use in studying protein-ligand interaction inside the living cell. We can use in-cell NMR spectroscopy to understand the functional role of membrane fusion proteins. By controlling the energy source and concentration of transported ligands, we should be able to observe different conformations of these membrane fusion proteins at various functional states.

It is certain that multidrug efflux transporters interfere significantly with the treatment of bacterial infections, by recognizing a number of structurally unrelated toxic compounds and actively extruding them from cells. Thus, these efflux transporters are clinically relevant and understanding the structural aspect of these proteins potentially can offer us for new drug targets. A more fundamental role for bacterial efflux transporters may be their contribution to the survival of bacteria during exposure to antimicrobial agents and toxic compounds. This may contribute to the survival of certain species within their ecological niche. While no efflux
transporter inhibitor has yet reach clinical practice, it is clear that this area of drug development offers a promising avenue and is considered to be of great clinical value.
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