Mechanistic Studies Of Drug Resistance Conferred By An ABC Transporter DrrAB

Han Zhang

Georgia State University

Follow this and additional works at: https://scholarworks.gsu.edu/biology_diss

Recommended Citation
Zhang, Han, "Mechanistic Studies Of Drug Resistance Conferred By An ABC Transporter DrrAB."
Dissertation, Georgia State University, 2013.
https://scholarworks.gsu.edu/biology_diss/125

This Dissertation is brought to you for free and open access by the Department of Biology at ScholarWorks @ Georgia State University. It has been accepted for inclusion in Biology Dissertations by an authorized administrator of ScholarWorks @ Georgia State University. For more information, please contact scholarworks@gsu.edu.
MECHANISTIC STUDIES OF DRUG RESISTANCE CONFERRED BY AN
ABC TRANSPORTER DrrAB

by

HAN ZHANG

Under the Direction of Dr. Parjit Kaur

ABSTRACT

Multi-drug resistance (MDR) has become a serious clinical problem for both cancer and infectious disease treatment. One of the leading causes of MDR is the drug efflux mediated by the ATP-binding cassette (ABC) super-family of proteins which are found among diverse groups of organisms. In our laboratory, we are interested in studying an ABC transporter DrrAB from *Streptomyces peucetius*. It confers resistance to two anticancer antibiotics doxorubicin and daunorubicin by its drug efflux function. Insights into the function of the DrrAB complex are expected to facilitate a better understanding of mechanisms of MDR.

The normal function of the DrrAB complex depends on the *cis* expression of both subunits, DrrA and DrrB. DrrA forms the nucleotide binding domain (NBD) and provides energy for drug translocation, while the DrrB protein functions as the transmembrane domain (TMD) and
forms the substrate translocation pathway. Studies on the detailed mechanisms of communication between DrrA and DrrB are critical for understanding the coupling of energy usage and substrate translocation. The present studies revealed the existence of two novel and functionally important modules in the C-terminal domain of DrrA that might be essential for conformational interplay between DrrA and DrrB during the catalytic cycle. One module present at the extreme C terminus of DrrA consists of two separate motifs, DEF and CREEM. CREEM motif together with its upstream region up to residue S319, interacts with the N-terminal cytoplasmic tail region of DrrB and forms an DrrA-DrrB interface, while the DEF motif regulates this interaction. The second novel module GATE is present 104 amino acids upstream of DEF. Based on our biochemical and structural analyses, we propose that GATE functions as a transducer of conformational changes (resulting from ATP binding) from DrrA to DrrB. Specifically, residue G215 present in the Gly-loop, through its close contacts with residues in the Walker A motif in the NBD, senses the conformational changes resulting from ATP binding to Walker A. Residue K227 in the GATE domain is then able to transduce those changes to the N terminus of DrrB via its interaction with S319 present in the extreme C terminus of DrrA.

INDEX WORDS: Multi-drug resistance, C-terminal domain, ABC transporter, Membrane protein
MECHANISTIC STUDIES OF DRUG RESISTANCE CONFERRED BY AN
ABC TRANSPORTER DrrAB

by

HAN ZHANG

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy
in the College of Arts and Sciences
Georgia State University
2013
MECHANISTIC STUDIES OF DRUG RESISTANCE CONFERRED BY AN
ABC TRANSPORTER DrtAB

by

HAN ZHANG

Committee Chair: Parjit Kaur
Committee: Phang C. Tai
John Houghton

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
May 2013
ACKNOWLEDGEMENTS

First and for most, I would like to thank my mentor Dr. Parjit Kaur, without whom this work could never be done. I am grateful for her well-rounded guidance and support throughout all these years’ Ph.D studies. Her understanding, sincerity and patience have encouraged me to overcome the obstacles and challenges that I encountered during my research. I could not remember how many times we sat down together and went through every single detail in a protocol when experiments didn’t work so well. The troubleshooting could be tough, but there were always laugh at some point which melted the difficulty and made me felt confident and optimistic. Her knowledge and vision have inspired me to not only become an experimenter but also more importantly an independent researcher. I am also thankful to my committee members Dr. Tai and Dr. Houghton for their very helpful directions and suggestions through my work. Their expertise and advice always enlightened my thoughts.

I also want to express my gratitude to Ms. Ling Wei, Dr. Prajakta Pradhan and Dr. Divya Rao and other previous fellows for their great help and training. My daily work was filled with joy because of friendly and cheerful lab members, Ms. Wen Li, Ms. Sadia Jannath and Mr. Chao Zhao. Thanks a lot for the help and fun that you all brought to me. You guys make me younger!

I would like to thank the members and staffs in Biology Core Facility and Department of Biology in Georgia State University for their technical support and assistance. I would also like to recognize the Molecular Basis of Disease fellowship for financial support and the opportunity to broaden my horizon through interdisciplinary research programs.

Last but not least, I would like to give my deepest appreciation for my parents. Thank you so much for your unconditional love, devotion and encouragement without which I could never had made it this far. My dearest husband and son, Mr. Xiaowei Liu and little Mr. Albert
Liu, with whom I share my life together, whenever it is happy or sad. Wherever life takes us, your embrace and love will always be my greatest comfort and my warmest home. I love you so much! I also would like to express my appreciation for my parents in-law for their selfless love and great support.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iv

LIST OF TABLES .................................................................................................................. x

LIST OF FIGURES .............................................................................................................. xi

INTRODUCTION ................................................................................................................. 1

MDR and ABC transporters .............................................................................................. 1

The structure and mechanism of ABC transporters ...................................................... 2

The DrrAB system .............................................................................................................. 4

Interactions between DrrA and DrrB are essential for assembly and function .......... 5

The C-terminal 132 amino acid domain of DrrA contains novel motifs involved in
interaction with DrrB and function of the DrrAB complex .............................................. 7

References ....................................................................................................................... 9

CHAPTER 1 .......................................................................................................................... 13

THE EXTREME C TERMINUS OF THE ABC PROTEIN DrrA CONTAINS .......... 13

UNIQUE MOTIFS INVOLVED IN FUNCTION AND ............................................. 13

ASSEMBLY OF THE DrrAB COMPLEX ................................................................. 13

1.1 Introduction .................................................................................................................. 13

1.2 Materials and methods ............................................................................................... 15

  Bacterial strains, plasmids, and antibodies .............................................................. 15

  Media and growth condition ..................................................................................... 16
Site-directed mutagenesis of DrrA ................................................................. 16

Mutagenesis of residues in the LDEVFL and CREEM motif ............................ 16

Deletion of the C terminus of DrrA ............................................................... 17

Doxorubicin resistance Assay ....................................................................... 18

Doxorubicin efflux assay ............................................................................... 18

Preparation and analysis of cell membranes ............................................... 19

ATP binding assay ....................................................................................... 19

Disulfide cross-linking ................................................................................. 20

Co-purification of DrrA and DrrB ................................................................. 20

Modeling analysis ....................................................................................... 21

1.3 Results ................................................................................................... 21

A glutamic acid-rich sequence at the extreme C terminus of DrrA is required for function of the DrrAB complex ................................................................. 21

The extreme C terminus of DrrA interacts with the N-terminal cytoplasmic tail of DrrB ........................................................................................................... 22

The C-terminal domain of DrrA contains conserved motifs ......................... 25

The conserved motifs in the extreme C terminus of DrrA are involved in function and assembly .......................................................................................... 27

Effect of mutations in LDEVFL and CREEM on expression of DrrA and DrrB . 27

Effect on ATP-binding to DrrA .................................................................... 28
Effect on doxorubicin resistance ............................................................................. 28

Effect on doxorubicin efflux .................................................................................... 29

Effect on DrrA-DrrB interaction ............................................................................. 30

Effect on co-purification of DrrA and DrrB ........................................................... 31

1.4 Discussion ............................................................................................................... 32

References .................................................................................................................... 37

CHAPTER 2 .................................................................................................................... 66

A NOVEL MODULE "GATE" IS ESSENTIAL FOR STABILITY AND ENERGY TRANSDUCTION IN DrrAB COMPLEX .............................................................................. 66

2.1 Introduction ........................................................................................................... 66

2.2 Material and methods ........................................................................................... 69

Bacterial, plasmids, and antibodies.......................................................................... 69

Growth conditions and Media .................................................................................. 70

Site-directed mutagenesis ......................................................................................... 70

Measurement of β-galactosidase activity ................................................................. 70

Doxorubicin resistance assay of GATE domain mutants ....................................... 71

Doxorubicin efflux assay of GATE domain mutants .............................................. 71

Modeling analysis of GATE domain mutants ......................................................... 73

2.3 Results .................................................................................................................... 73

Identification of the GATE domain in DrrA and other ABC proteins ................. 73
Conserved glycines (G215 and G221) in the GATE domain are critical for stability of the DrrAB complex ................................................................. 74

Several other conserved residues (E201, G221, L226, K227, and G231) in the GATE domain are also critical for the function of the DrrAB complex .............. 76

Role of the conserved residues of the GATE domain in ATP binding .............. 78

The GATE domain does not show direct interaction with the N-terminus of DrrB ........................................................................................................ 79

Insights from the structural analysis of the GATE domain in MalK and DrrA .... 79

2.4 Discussion .............................................................................................................. 82

A proposed model for the role of the conserved motifs in the C-terminal domain of DrrA ........................................................................................................ 87

References .................................................................................................................. 89

GENERAL DISCUSSION .............................................................................................. 108

References .................................................................................................................. 112
LIST OF TABLES

CHAPTER 1

Table 1.1 Effect of mutations in the LDEVFL or CREEM motif on doxorubicin resistance...... 41

Table 1.2 Effect of mutations in the LDEVFL or the CREEM motif on doxorubicin efflux...... 42

CHAPTER 2

Table 2.1 Effect of mutations in the GATE domain on doxorubicin resistance.............................94
LIST OF FIGURES

GENERAL INTRODUCTION

Fig. A The ATP switch model of ABC exporters................................................................. 6

CHAPTER 1

Fig. 1.1 Schematic representation of the conserved motifs in DrrA.................................43
Fig. 1.2 Disulfide cross-linking between S319C in DrrA and S23C or C260 in DrrB..........45
Fig. 1.3 Disulfide cross-linking between T311C, S302C, S287C, S253C, or S232C in DrrA and S23C in DrrB.................................................................46
Fig. 1.4 Sequence alignment of the C terminus of DrrA and its prokaryotic or eukaryotic homologs...........................................................................................................47
Fig. 1.5 Effect of mutations in the LDEVFL motif on expression of DrrA and DrrB........48
Fig. 1.6 Effect of mutations in the LDEVFL or CREEM motif on ATP binding.................49
Fig. 1.7 Effect of LDEVFL mutations on disulfide cross-linking between DrrA(S319C) and DrrB(S23C).........................................................................................51
Fig. 1.8 Effect of mutations in the LDEVFL motif on co-purification of DrrA and DrrB.............................................................54
Fig. 1.9 Structure modeling of DrrA.. .............................................................................56
Fig. S1.1 Disulfide cross-linking between A323C or E325C in DrrA and S23C in DrrB........58
Fig. S1.2 ClustalW alignment of the C terminal sequence (residues 199-315) of DrrA with the C-terminal sequences of bacterial homologs identified by BLAST search.................................................59
Fig. S1.3 ClustalW alignment of the last 132 amino acids of DrrA with the C-terminal sequences of NBDI of eukaryotic homologs identified by TC-Blast search. .........................60
**Fig. S1.4** ClustalW alignment of the last 132 amino acids of DrrA with the C-terminal sequences of NBDII of eukaryotic homologs identified by TC-BLAST search. ........................................61

**Fig. S1.5** Effect of LDEVFL or CREEM mutations on doxorubicin efflux ..........................62

**Fig. S1.6** Docking analysis of the predicted structures of DrrA and DrrB ............................64

**Fig. S1.7** A model showing various interactions between DrrA-DrrB and DrrA-DrrA during different stages of the catalytic cycle .................................................................65

**CHAPTER 2**

**Fig. 2.1** ClustalW amino acid sequence alignment of the Glycine Loop And Transducer Element (GATE) domain of DrrA with other ABC transporters .................................................................95

**Fig. 2.2** The tertiary structure of the GATE domain of DrrA and other ABC homologs ................96

**Fig. 2.3** Effect of point mutations in GATE domain on DrrA and DrrB expression ..........................97

**Fig. 2.4** β-galactosidase activity of the drrA-lacZ and drrAB-lacZ translational fusions ..........................98

**Fig. 2.5** The effect of point mutations in the GATE domain on doxorubicin efflux by the DrrAB transporter ..................................................................................................................99

**Fig. 2.6** The effect of the GATE domain mutations on ATP-binding to the DrrAB complex ............100

**Fig. 2.7** Disulfide cross-linking between cysteines introduced in the GATE domain of DrrA and the N-terminus of DrrB ..................................................................................................101

**Fig. 2.8** Structural analysis of MalK and DrrA .............................................................................102

**Fig. 2.9** A model showing 2-way communication between DrrA and DrrB mediated by the N-terminus of DrrB and different motifs in DrrA .............................................................................103

**Fig. S2.1** Effect of E201D, L205V, G221A or G221S mutation on doxorubicin efflux ..............104

**Fig. S2.2** Effect of L226V or K227R mutation on doxorubicin efflux ........................................105
Fig. S2.3 Effect of G231A or G231S mutation on doxorubicin efflux.................................106

Fig. S2.4 Effect of E201Q, G215A, G215S or G215P mutation on doxorubicin efflux...........107
INTRODUCTION

MDR and ABC transporters

Multi-drug resistance (MDR) has become a serious clinical problem for both cancer and infectious disease treatment in the last few decades. It results in resistance to a broad variety of structurally and functionally unrelated drugs and antibiotics [1]. Bacterial cells employ multiple mechanisms to evade antibiotics, which include enzymatic modification of the drug, mutagenesis of drug targets, altered membrane permeability, and enhanced efflux pump expression [2]. Of these, drug efflux by dedicated membrane transporters is the most commonly seen mechanism for multi-drug resistance. Most drug efflux proteins can be classified into five distinct protein families: the resistance-nodulation-cell division (RND), major facilitator (MF), staphylococcal small multidrug resistance (SMR), multidrug and toxic compound extrusion (MATE), and ATP-binding cassette (ABC) families. The first four families are driven by proton (and sodium) motive force, whereas ATP hydrolysis drives drug efflux in ABC transporters [2].

The ABC super-family of proteins is found among diverse groups of organisms. They play pivotal roles in multiple biological processes including the import of different nutrients and export of various molecules. Nevertheless, there are some ABC members that are involved in translation elongation and DNA repair [3]. One of the most extensively studied MDR-associated ABC transporter is the P-glycoprotein (ABC sub-family B member 1, ABCB1 or MDR1) found in human cancer cells. MDR1 confers resistance to multiple drugs including doxorubicin, daunorubicin, colchicine, vincristine, vinblastine, rhodamine 123 and hoechst, etc [1]. The prokaryotic analogs of MDR1 include LmrA from Lactococcus lactis, the lipid flippase MsbA from Escherichia coli (E. coli) and Sav1866 from Staphylococcus aureus. Recent knowledge about
the structure and function of these transporters has provided profound insights into the mechanisms of ATP-dependent drug efflux [4-7].

The structure and mechanism of ABC transporters

The basic structure of an ABC transporter consists of two transmembrane domains (TMDs) and two cytoplasmic nucleotide-binding domains (NBDs) [8]. The two TMDs together form the substrate translocation pathway, while the two NBDs form a head-to-tail interface which binds and hydrolyzes ATP to provide energy for substrate influx or efflux [8]. ABC transporters in eukaryotic cells commonly occur as a single large protein that contains all four domains. In prokaryotes, however, most ABC transporters contain the TMD and the NBD on separate subunit. These subunits form a tetramer structure that is very similar to the eukaryotic ABC transporters [1].

The NBD of an ABC protein contains all the characteristic motifs essential for ATP-binding and hydrolysis, which include Walker A, Q-loop, ABC signature, Walker B, D-loop and the Switch. The tertiary structure of the NBD exhibits two domains: the larger domain referred to as the ‘RecA-like’ domain that contains the Walker A (GxxGxGKS/T, where x represents any amino acid) and Walker B (ϕϕϕϕD, where ϕ stands for a hydrophobic residue) motifs, and the smaller domain termed ‘helical domain’ which consists of three to four helices and the ABC signature (LSGGQ) motif [3]. The helical domain is sometimes also called the structurally diverse region (SDR), which may be involved in selectivity of particular substrates and the corresponding communication between the NBD and TMD [9]. A motif called the Q-loop connects the RecA-like domain and the helical domain. In comparison to NBDs, TMDs from different ABC transporters share less similarity in their amino acid sequences. Typically, each TMD contains
six to ten transmembrane α-helices, and a complete substrate translocation pathway requires the dimerization of TMDs.

One open question about ABC transporter function concerns the mechanism underlying the power stroke of substrate translocation. A widely accepted model known as the ‘ATP switch model’ for the transport cycle of an ABC exporter is shown in Fig. A [10]. Prior to the binding of the substrate to the inner leaflet of the membrane, the NBD dimer is in an open or resting state and TMDs are in the inward-facing conformation. The translocation process is initiated by the binding of substrate to its binding site in TMDs (cyan and red), which increases the affinity of NBDs (blue and orange) for ATP. Two molecules of ATP then bind to the ATP-binding pockets in a cooperative manner, which consequently induces the closed form of the NBD dimer. The resulting conformational changes promote the transfer of TMDs from inward-facing to outward-facing conformation followed by extrusion of the substrate. In the third step, ATP is hydrolyzed followed by the sequential release of phosphate and ADP. Consequently, the transporter is restored to its resting state. A large body of literature supports this model [8, 10, 11], however the detailed mechanisms involving the signaling between the NBD and the TMD as well as the coupling of energy usage and substrate translocation still remain elusive. It is generally accepted that conformational changes generated by ATP binding and/or hydrolysis are transmitted from the NBD to TMD through domain-domain interactions at the shared interface. One of the earliest studies that investigated such interactions was carried out on the maltose transport system, MalFGK₂. Genetic studies showed that several functionally drastic mutants in a hydrophilic cytoplasmic loop in both TMDs, MalF and MalG, could be suppressed by mutations in the helical domain of MalK (the NBD component of the transporter), implying the importance of association between these two domains [12]. A conserved sequence called the EAA motif was identi-
fied in the above hydrophilic cytoplasmic loop in MalF and MalG, which was subsequently found to be present among several binding protein-dependent ABC importers [12]. A similar, though not identical, motif termed the EAA-like motif is present in ABC exporters [13], as described later. The focus of the present study is an ABC drug transporter DrrAB. This study reports identification of novel motifs in the ABC component DrrA and illustrates their role in interaction with the N terminus of DrrB (which contains the EAA-like motif) and their possible role in energy transduction between DrrA and DrrB.

**Fig. A** The ATP switch model of ABC exporters. Step I, substrate binding to the pocket formed by the TMDs induces conformational changes in the NBDs, resulting in their increased affinity for ATP. Step II, two molecules of ATP bind to the NBDs interface and induce the closed NBD dimer. As a consequence, the resulting conformational changes trigger the TMDs to transfer from inward-facing to outward-facing conformation and the substrate is extruded. Step III, ATP hydrolysis promotes further conformational changes in the TMDs. Finally, the release of phosphate and then ADP restores the transporter to the resting state.

**The DrrAB system**

*Streptomyces peucetius*, a gram-positive bacterium, produces two anti-cancer chemotherapeutic agents, doxorubicin and daunorubicin, and is self-resistant to these drugs. The self-resistance is conferred by an ABC efflux pump consisting of two proteins: the doxorubicin resistance protein A (DrrA) and B (DrrB). They are encoded by two open reading frames, *drrA* and *drrB*, within a gene cluster termed DNR/DXR [14]. After sub-cloning these two genes in an
E. coli expression vector, DrrA and DrrB could be expressed to form a functional transporter, which was shown to facilitate doxorubicin efflux and confer doxorubicin resistance [15].

DrrA is a 36-kDa protein consisting of 330 amino acid residues. It is a hydrophilic peripheral membrane protein, which was shown to bind ATP or GTP in a Mg\(^{2+}\)-dependent manner [15]. Sequence alignment of DrrA and NBDs from other ABC transporters suggested that DrrA possesses all the conserved motifs required for ATP catalysis in its N-terminal region (1-198 residues) [16]. In a recent research work conducted in our lab, fluorescence resonance energy transfer (FRET) studies confirmed the head-to-tail conformation of the DrrA dimer [16]. DrrB contains 283 residues with predicted molecular weight of 30.6 kDa. It is highly hydrophobic and is localized in the membrane [15]. Gene fusion studies showed that DrrB contains eight transmembrane helices with both the N and the C termini localized to the cytoplasm [17]. The complete DrrAB transporter is proposed to be a tetramer consisting of two DrrA and two DrrB subunits [18].

**Interactions between DrrA and DrrB are essential for assembly and function**

Previous studies have shown that DrrA plays an important role in stability of DrrB and that it may function as a chaperone molecule [18]. When expressed alone in E. coli cells, DrrB is improperly assembled and is degraded [18]. When co-expressed with *drrA* either in *cis* or *trans*, the normal expression level of DrrB in the membrane is restored [18]. Interestingly, DrrA fails to bind ATP in the absence of DrrB, indicating that DrrB is necessary for DrrA to acquire active conformation [18]. These data strongly suggest that DrrA and DrrB depend on each other for normal stability and function. The finding that DrrA and DrrB proteins confer doxorubicin resistance only when the two proteins are expressed in *cis* in a translationally coupled manner further supports the notion that DrrA and DrrB proteins may be required to co-fold in order to
form a functional efflux pump [19]. These observations raise the questions: are there specific domains present in these two proteins that mediate the association between them and thus the proper assembly of the complex? Are these domains also involved in signal transduction between the two proteins during the drug translocation process?

Important insights were obtained by using chemical cross-linking approaches. Utilizing a heterobifunctional cysteine to amine cross linker GMBS, it was revealed that a cysteine substitution mutant S23C in the N-terminal cytoplasmic tail of DrrB (residue 1-53) interacts with a region in DrrA. An conserved motif called the EAA-like motif (containing the consensus sequence GE_{1..3}A_{3..5}R/K..G_{7}) was subsequently identified in this region of DrrB based on its sequence similarity to the conventional EAA motif found in ABC importers [16]. Such a motif has also been recently identified in TMDs from other ABC exporters [16]. In an attempt to explore the regions in DrrA that interact with S23C in the N-terminus of DrrB, a cysteine scan of the NBD (residue 1-198) of DrrA was carried out. Disulfide cross-linking studies showed that S23C interacts with Y89C introduced in the Q-loop of DrrA. These studies, therefore, defined an interaction interface between DrrA and the N-terminus of DrrB. Another important interaction interface is formed between the extreme C terminus of DrrA and the N terminus of DrrB, which is described in the next section. Interestingly, it was also found that Y89C traps DrrA in the homodimeric state, indicating that Q-loops from the opposing subunits are in close proximity in the closed conformation of the NBD dimer of DrrA [16]. However, the conformation of the Y89C-Y89C dimer was shown to be different from that in the strain containing both Y89C and S23C. Using disulfide cross-linkers of different arm lengths, it was shown that the presence of S23C in DrrB produces changes in conformation of the Y89C-mediated DrrA dimer, making it more rigid and probably more potent for ATP-binding. It was thus proposed that the NBD dimer is normal-
ly in a flexible contact until a conformational change in DrrB (mimicked by S23C substitution in this situation) produces a consequent change in DrrA that fixes the NBD dimer in the closed state. This conformational change may resemble the transient state in which the signal of doxorubicin binding to DrrB is transmitted to the NBD and induces formation of the closed NBD dimer [16].

**The C-terminal 132 amino acid domain of DrrA contains novel motifs involved in interaction with DrrB and function of the DrrAB complex**

Recently a spate of studies has revealed that the C-terminal extensions of the NBD components of ABC proteins may contain important functions, which makes this region emerge as an essential regulatory apparatus for substrate translocation [20-28]. The amino acid sequence of this region of ABC proteins is not highly conserved except in closely related proteins, however a structural similarity has been observed in these regions based on the 3-dimensional analysis of some ABC proteins [20-22]. DrrA also contains a unique C-terminal extension that covers the last 132 amino acid residues (residue 199-330). Since this region is organized separately from the conventional NBD motifs which are present in the N-terminal domain of DrrA, it is reasonable to propose that it carries out a specific function other than direct ATP binding and hydrolysis.

Since the function of the C-terminal region of DrrA has never been studied before, the present study set out to find out whether this region contains motifs that are specifically important for function of the transporter. In this dissertation, three novel motifs, GATE, DEF (referred to as LDEVFL in Chapter 1) and CREEM, were identified in the C-terminal 132 amino acid domain of DrrA. Of these, DEF (302-SLDEVFLALTGH-313) and CREEM (321-EEAAEEEEKVA-330), present at the extreme C terminus of DrrA, were found to be conserved in other prokaryotic and eukaryotic proteins of the DRA family of ABC proteins to which the
DrrAB system belongs. The most noteworthy among these are the ABCA subfamily proteins 1, 2, 3 and 8, which are primarily involved in lipid trafficking and homeostasis in mammalian cells [29]. Interestingly, disulfide cross-linking studies showed that the CREEM motif, together with its immediate upstream region up to residue S319, exhibits strong interaction once again with the N-terminal cytoplasmic tail region of DrrB, whereas the DEF motif regulates this interaction and therefore possibly the assembly and function of the DrrAB complex. The present study, therefore, defines a second interaction interface between the extreme C terminus of DrrA and the N terminus of DrrB.

The third novel motif referred to as ‘Glycine-loop And Transducer Element’ (GATE) was found to be conserved not only among close homologs of the DRA family, but is also found in diverse ABC proteins, including MalK, ModC, BtuD, Pgp, Cystic fibrosis transmembrane conductance regulator (CFTR), MsbA, and Sav1866. Based on our biochemical analyses, three highly conserved glycines in this motif were found to be indispensable for conferring the necessary flexibility for proper integrity and function of the complex. The analysis of the MalK crystal structure and the DrrA model suggested that one of these glycines, G215, forms hydrogen bonds with important residues in the Walker A motif which have direct interactions with ATP, indicating that G215 may be involved in ATP binding and/or catalysis. Additional structural studies further suggest that GATE may function as a transducer of conformational changes during the catalytic cycle of DrrAB the complex. Specifically, G215 may be able to sense the conformational changes resulting from ATP binding to Walker A, and another essential residue K227 in the GATE domain may then be able to transduce those changes to DrrB through its interaction with S319 in the extreme C terminus of DrrA.
The present work, therefore, revealed the presence of two distinct functional modules in the C-terminal region of DrrA and provided valuable insights for two of the most important aspects underlying the mechanism of the DrrAB transporter. First, these studies showed that the two motifs present in the extreme C terminus of DrrA are involved in interaction with DrrB, which may facilitate proper assembly and function of the complex. Second, the GATE domain located between the N- and the C- terminal domains of DrrA may be crucial for mediating signal transduction and communication between DrrA and DrrB during catalysis. The conservation of these motifs among other members of the ABC family suggests that they may play similarly important roles in the other ABC proteins.

References


CHAPTER 1
THE EXTREME C TERMINUS OF THE ABC PROTEIN DrrA CONTAINS UNIQUE MOTIFS INVOLVED IN FUNCTION AND ASSEMBLY OF THE DrrAB COMPLEX

1.1 Introduction
Membrane proteins play vital roles in critical biological systems, which include solute transport, signal transduction, and energy conservation. Loss of function, or misassembly, of membrane proteins is frequently associated with severe medical conditions. However, due to the many hurdles involved in the study of these proteins, assembly and function of membrane protein complexes is still poorly understood. Moreover, diverse mechanisms and factors (both extrinsic and intrinsic) can contribute to localization and assembly of membrane proteins (1,2), thus making the analysis of this process quite challenging. We are interested in elucidating the function of the DrrA and DrrB proteins of *Streptomyces peucetius*, which together form an ATP-driven efflux pump for doxorubicin and daunorubicin, two antibiotics used in the chemotherapy of cancer. DrrA belongs to the ABC family of proteins and forms the catalytic subunit (3,4), while DrrB is an integral membrane protein containing eight transmembrane α-helices (5). Therefore, in this system, the catalytic function and the membrane transport function are present on separate subunits, which form a tetrameric complex and carry out doxorubicin efflux. Some other ABC transporters, such as P-glycoprotein (Pgp) and cystic fibrosis transmembrane conductance regulator (CFTR), contain two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) fused together into a single, large polypeptide (6).

Previous studies have shown that DrrB is improperly assembled in the absence of DrrA. In this situation, DrrB is either quickly degraded (7) or, if overexpressed, it severely inhibits
growth (4), implying that interaction with DrrA is essential for stability, and perhaps also for proper conformation of DrrB (7). Recent studies have shown that DrrA and DrrB proteins confer doxorubicin resistance only when the two proteins are expressed in \textit{cis} in a translationally coupled manner (8). It is thus likely that the DrrA and DrrB proteins may be required to co-fold for the formation of a fully functional DrrAB complex, and translational coupling may facilitate this interaction. This raises the question: is a specific interaction between DrrA and DrrB involved in the assembly process? Here we show that the C terminus of DrrA indeed contains two novel motifs, which play a critical role in function and assembly of the DrrAB complex. One motif present at the extreme C terminus of DrrA is rich in glutamic acid residues and is termed the ‘C-terminal \text{Regulatory E-rich Motif (CREEM)}’ in this study. The second motif, termed LDEVFL, is located upstream of the CREEM motif. Interestingly, by homology sequence comparisons, these two motifs were found to be conserved in other prokaryotic and eukaryotic ABC proteins, the most noteworthy among these being the ABCA subfamily proteins 1, 2, 3, and 8, as well as Ced-7, which belong to the same family (DRA) of ABC proteins as DrrAB (9). ABCA proteins are involved in lipid efflux in mammalian cells, and defects in the function or assembly of proteins of this subfamily are associated with severe medical conditions, including Tangier and Alzheimer’s diseases (10,11).

Recently, a spate of investigations has reported that the C-terminal domains of some ABC proteins may be associated with specialized functions. The amino acid sequence of the C-terminal domains is, however, only conserved among closely related proteins. For example, a vast majority of binding protein-dependent sugar transport systems harbors a \~150 amino acid long C-terminal extension containing three conserved regulatory motifs termed RDM1-3 (12,13). In MalK of \textit{Escherichia coli}, this region binds MalT and EII\text{glc} and plays a key role in regulation
of expression (14,15) and in inducer exclusion (12,16). Of the non-sugar ABC transporters, the C-terminal regulatory domain present in ModC of the molybdate/tungstate transporter (Ma-ModBC) in *Methanosarcina acetivorans* binds molybdenum and is involved in trans-inhibition of the ATPase activity, which results in a decrease of the transport rate in response to an increase in concentration of the substrate in the cytoplasm (17). Similarly, C-terminal extensions present in MetN of the MetNI system (18) and in Wzt of the Wzt/Wzm system of *E. coli* are able to bind their respective pump substrates (19,20). Crystal structure analysis suggests that the C-terminal domains of these proteins contain a similar β-sheet fold, though they contain diverse amino acid sequences and perform different functions in ABC transporters.

In the studies described here, we report that the CREEM and LDEVFL motifs present in the extreme C terminus of DrrA are critical for function of the DrrAB complex. We also show that this region of DrrA forms the point of contact with the N-terminal cytoplasmic tail of DrrB, thus leading to the proposal that the major role of the CREEM and LDEVFL motifs may be in assembly of the DrrAB complex. Interestingly, a 33 amino acid region in the C terminus of DrrA, encompassing residues in the LDEVFL motif, was also found to be involved in homodimerization of DrrA. The significance of these two interactions, both localized to the C-terminal end of DrrA, in protein assembly is discussed.

1.2 Materials and methods

**Bacterial strains, plasmids, and antibodies**- The bacterial strains used in this study were *E.coli* TG1, N43, LE392ΔuncIC, HMS174, and XL1-Blue. The plasmids used in this study include pDx101 (*drrAB* in pSU2718) and pDx119 (*drrAB* in pET 16b). Various substitutions and deletions were created in the *drrA* and *drrB* genes in these plasmids. Rabbit polyclonal antibodies,
generated against DrrA and DrrB previously (4), were used for Western blot analysis. Anti-SecY antibody was provided by the laboratory of Dr. P.C. Tai.

**Media and growth condition**- For doxorubicin efflux experiments, cells were grown in TEA medium [50 mM triethanolamine HCl (pH 6.9), 15 mM KCl, 10 mM(NH₄)₂SO₄, 1 mM MgSO₄] supplemented with 0.5 % (w/v) glycerol, 2.5 µg/ml thiamine, 0.5 % (w/v) peptone, and 0.15 % (w/v) succinate at 37°C(21). For site-directed mutagenesis, XL1-Blue cells were grown at 37°C in NYZ + broth [pH 7.5, 1% (w/v) casein hydrolysate, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl] supplemented with 12.5 mM MgCl₂, 12.5 mM MgSO₄ and 0.4% (w/v) glucose (La Jolla, CA). For all the other experiments, cells were grown in LB medium. Chloramphenicol was added to 20 µg/ml, and ampicilin was added to 75 µg/ml, where needed.

**Site-directed mutagenesis of DrrA**- A QuikChange multisite-directed mutagenesis kit (La Jolla, CA) was used to create various mutations in the *drrA* gene. The strategy involved the use of complementary primers that incorporated the change at the required position.

**Mutagenesis of residues in the LDEVFL and CREEM motif**- pDx101 was used as a template. Primers were designed as described before (22). In the LDEVFL motif, conservative point mutations at positions 303 and 304 were created, which were named L303V and D304N, respectively. Besides, two sets of triple alanine substitutions were performed at 306, 307, 308 and 310, 311, 312, resulting in V306A/F307A/L308A and L310A/T311A/G312A, respectively. Deletion of the LDEVFL motif was obtained by using a pair of primers consisting of 15 flanking bases on each side of the sequence to be deleted, and the resulting clone has been designated ΔLDEVFL. In the CREEM motif, 3, 4 or 5 glutamic acid residues, present within the last 10 amino acid region of DrrA, were altered to glutamine, aspartic acid or glycine residues. The obtained mutants in-
include E(321, 322, 325)D, E(321, 322, 325)G, E(321, 322, 325, 326)Q and E(321, 322, 325, 326, 327)G, which are referred to as 3E-3D, 3E-3G, 4E-4Q and 5E-5G, respectively.

Single cysteine substitutions in DrrA- pDx101 containing a single cysteine substitution in *drrB* at position 23(S23C) was used as the template (22). Single cysteine substitution mutants were created at amino acid positions 325, 323, 319, 311, 302, 287, 253 or 232 in DrrA in this clone.

**Deletion of the C terminus of DrrA**- This was achieved by removing 27 bases (960-987) from the 3’ end of *drrA* while retaining the last 3 bases of the sequence in order to maintain translational stop/start overlap with *drrB*. An *XhoI* site was first introduced in the 3’ end of *drrA* by substituting 3 bases at positions 963, 966 and 968 in pDx101. These changes did not alter the coding sequence of *drrA*. The sequence of the primers used was:

- UP- *XhoI*: 5’-GCCGATGACCGCTCGAGGGCATGACGACGTCCCCCGGCACCGTGGAATCCACGACCCCTGAGCGGTAGC-3’
- DN- *XhoI*: 5’-GCTTCTTCCCTCGAGCGGTTCATCGGC-3’
- GGTCATCGGC-3’.

This construct was designated pDx137. pDx137 DNA was then digested with *XhoI* (at 960th nucleotide in *drrA*) and *FseI* (at 84th nucleotide in *drrB*) to remove 139 bp fragment from the intergenic region of *drrAB*. A fragment corresponding to this region was then synthesized by mutually primed synthesis using single-stranded oligonucleotides. The oligonucleotides, which are shown below, were designed such that 27 base pairs of *drrA* will be deleted from the synthesized fragment. The flanking regions contained *XhoI* and *FseI* restriction sites.

- Spacer27delUP:
  5’GCCGATGACCGCTCGAGGGCATGACGACGTCCCCCGGCACCGTGGAATCCACGACCCCTGAGCGGTAGC-3’
Spacer27delDN:

\[ \text{5'}CCGTCGC}CGGGCCG\text{CGGC}TTCA\text{CCCGCGACAGC}\text{ACC}TG\text{CGGACAGCTGAC} \]
\[ \text{CGCTC}\text{ACA}GGGGTGTGGATT3'}. \]

The 112 bp fragment was digested with XhoI and FseI and then ligated to pDx137 DNA that had been digested with the same enzymes. The resulting clone has been designated \( \Delta \text{CREEM}. \)

**Doxorubicin resistance Assay**- Doxorubicin resistance assays were carried out as described earlier (23). Briefly, the indicated plasmids were transformed into *E.coli* N43 cells which are doxorubicin sensitive. A single colony was incubated in 5ml LB containing desired antibiotic for 8 hours. 1 µl of the above cells were streaked on M9 plates with a top layer containing 0, 4, 6, 8 or 10 µg/ml doxorubicin. Plates were covered with foil because doxorubicin is light sensitive. Growth was recorded after incubation of plates at 37°C for 24 hours.

**Doxorubicin efflux assay**- The efflux assay was carried out according to the protocol previously developed in this laboratory (Sharma and Kaur, unpublished). Briefly, *E. coli* LE392\( \Delta \text{unc} IC \) cells (24) were transformed with the indicated plasmids; the cells were grown to mid-log phase (optical density \(600 \text{ nm} = 0.6\)) and induced with 0.1 mM IPTG for 1 hour, harvested and washed with TEA twice and resuspended in 1 ml of TEA. 10 µl of the cell suspension from above was incubated in 3 ml of TEA medium containing 10 µM doxorubicin and 5 mM 2, 4-dinitrophenol (DNP) for 11 hours at 37°C. The loaded cells were washed twice with 0.1 M MOPS buffer, pH 7.0, containing 2.0 mM MgSO\(_4\) and resuspended in 3 ml of MOPS buffer. The fluorescence spectra were recorded on an Alphascan-2 spectrofluorometer (Photon Technology International, London, Ontario, Canada). The excitation wavelength for doxorubicin was 480 nm and emission was monitored at 590 nm. The excitation and emission slit widths were set at 0.75 and time
based script was run. After an initial recording of fluorescence for 100 seconds at 37°C, energy was provided in the form of 20 mM glucose and recording was continued for an additional 400 seconds.

**Preparation and analysis of cell membranes** - 50 ml *E.coli* TG1 cells containing the indicated plasmids were grown to mid-log phase and induced with 0.1 mM IPTG. Growth was continued for an additional 3 h at 37°C. The membrane fraction was prepared as follows: cells were spun down and resuspended in 10 ml buffer A [25 mM Tris-Cl (pH7.5), 20% glycerol, 2 mM EDTA (pH8.0), 1 mM DTT] and passed through a French Press cell at 16,000 psi, followed by centrifugation at 10,000 g at 4°C for 30 min to remove unbroken cells. The supernatant was centrifuged at 100,000 g at 4°C for 1 hour. The pellet was resuspended in 10 ml buffer A, washed twice, and finally resuspended in 100 µl buffer A. The samples were heated at 55°C for 10 min and loaded on 10% SDS-polyacrylamide gel. The proteins were transferred onto the nitrocellulose membrane for 2h at 55V at room temperature, followed by Western blot analysis or autoradiography. Densitometric scanning of the bands in both the autoradiogram and the Western blots was performed by Multi Gauge V2.3 (FUJIFILM).

**ATP binding assay** - Photolabeling of DrrA with [%α-32P] ATP was carried out in membranes containing wild type DrrAB or DrrAB bearing mutations in DrrA (23). ATP binding assay was carried out in a 100 µl reaction system containing buffer A, 0.1 mg membrane protein, 10 µM ATP (pH7.5), 10 µCi α-32PATP, 35 µM doxorubicin and 5mM MgCl2. The reaction was exposed to UV light at 254 nm on ice for 30 min, followed by protein precipitation by 10% ice-cold TCA on ice for 30 min. Protein was recovered by centrifugation at 14,000 g for 15 min and the pellet was resuspended into 20 µl of 4X Laemmli sample buffer and 5 µl 1M unbuffered Tris. The samples were resolved by polyacrylamide gel electrophoresis and transferred onto nitrocellulose mem-
brane, as described in the previous section. The membrane was air-dried and exposed to X-ray film at -70°C overnight, followed by autoradiography. The same blot was then examined by Western blot using anti-DrrA antibody.

**Disulfide cross-linking-** A 100 μl reaction volume containing 250 μg of membrane protein in 0.1M phosphate buffer pH 7.4 (0.1M of Na₂HPO₄ and 0.1M NaH₂PO₄) was treated with thiospecific reagents including copper phenanthroline (CuPhe, 3 mM CuSO₄/9 mM 1, 10-Phenanthroline) or 1 mM dithio-bis-maleimidoethane (DTME, Pierce Chemicals). The cross-linking reaction was carried out at room temperature for 30 min and stopped by adding 4X Laemmli sample buffer. A 25 μl portion (50 μg of membrane protein) of the reaction mixture was then analyzed by 10% SDS-polyacrylamide gel, followed by Western blot analysis using anti-DrrA or anti-DrrB antibodies.

**Co-purification of DrrA and DrrB-** pDx119-derived clones bearing different mutations in DrrA were used for co-purification of DrrAB proteins. HMS174 cells containing the indicated plasmids were grown to mid-log phase at 37°C. The expression of DrrAB was induced with 0.1 mM IPTG at 20°C and growth was continued for 16 hours at 20°C. DrrAB proteins were solubilized from 5 mg membrane fraction with 5 ml solubilization buffer [50 mM Tris-Cl (pH7.5), 1% n-dodecyl-β-D-maltoside (DDM), 1μl 14.3 M β-mercaptoethanol, 20% glycerol, 200 mM NaCl] on ice for 1h. After centrifugation at 100,000 g at 4°C for 1 hour, the supernatant was loaded on a Ni-NTA column at 4°C. The DrrAB proteins were eluted with the buffer containing 50 mM Tris-Cl (pH7.5), 300 mM NaCl, 20% glycerol, 0.05% DDM and 500 mM imidazole at 4°C. The purified proteins were resolved on 12% SDS-polyacrylamide gels and analyzed by Western blot using anti-DrrA or anti-DrrB antibodies.
Modeling analysis- AMMP protein structure modeling software ((26), http://asterix.cs.gsu.edu/ammp.html) was used for homology modeling of DrrA and its mutants using the crystal structure of MalK (PDB ID: 2R6G; (25)) as a template. Modeling analysis of the membrane protein DrrB was carried out using PHYRE protein fold recognition server ((27), http://www.sbg.bio.ic.ac.uk/phyre/) since DrrB is not homologous to the MalG or MalF protein or another crystallized membrane protein of the ABC family. The models of DrrA and DrrB complexes were then created by using Rosetta Dock protein-protein docking server ((28), http://rosettadock.graylab.jhu.edu/). Please note that docking analysis requires that the investigator provide a reasonable starting position for docking of two proteins. Based on the biochemical data presented in this paper, we provided the starting position where the relevant portion of the C terminus of DrrA and the N terminus of DrrB faced each other. Using this starting position, the Rosetta Dock server modeled the wild type and mutant DrrAB complexes. PyMOL Molecular Graphics System ((29), http://www.pymol.org/) was used to view all the predicted structures and for presentation of the models.

1.3 Results

A glutamic acid-rich sequence at the extreme C terminus of DrrA is required for function of the DrrAB complex- DrrA belongs to the ABC family of proteins and contains all the known conserved motifs (including Walker A, Walker B, Q-loop, signature, and switch motifs) required for the catalytic function of ABC proteins. These motifs are confined to the N-terminal domain of DrrA (residues 41-198)(23)(Fig. 1.1A), while the C-terminal domain (residues 199-330) is not known to contain any conserved motifs involved in function or assembly. In this study, we identified a glutamic acid-rich sequence (EEAAEEEKVA) at the extreme C terminus of DrrA, which suggested the possibility that the negatively charged residues present in this region may be in-
volved in interaction between DrrA and DrrB. To determine the role of this region, last 9 amino acids (residues 321-329) of DrrA were deleted, but the overlapping sequence between the translational stop of DrrA and the start of DrrB was retained. This truncation reduced the expression of DrrA and DrrB to roughly 55% and 78% of the wild type, respectively (Fig. 1.1B, lane7, and Figs. 1.1C and 1.1D). Doxorubicin resistance was severely compromised in this strain (Table 1.1). One explanation for these data could be that this region may play a role in stability of DrrA; however, when site-directed substitutions of 3, 4 or 5 glutamates with aspartates, glycines or glutamines were carried out (resulting in 3E-3D, 3E-3G, 4E-4Q and 5E-5G, as described in Methods), the expression level of DrrA and DrrB remained about the same as in wild type cells (Fig. 1.1B, lanes3-6 and Figs. 1.1C and 1.1D). Doxorubicin resistance in the strain containing 5E-5G mutation was found to be drastically affected, whereas it was only partially compromised in strains containing substitutions of three or four glutamates (Table 1.1). Together, the deletion and mutagenesis data indicate that the glutamic acid-rich region in the extreme C terminus of DrrA is important for function, and suggest that it may play a role in DrrA-DrrB interaction and assembly of the complex. This region has been termed ‘C-terminal Regulatory E-Rich Motif’ (CREEM)’ in this study. Further characterization of this motif is described in later sections.

**The extreme C terminus of DrrA interacts with the N-terminal cytoplasmic tail of DrrB** - To determine if the extreme C terminus of DrrA interacts with DrrB, disulfide cross-linking experiments were performed. Previous studies, using a cysteine to amine cross linker GMBS, have shown that the N–terminal cytoplasmic tail of DrrB (residues 1-53) is the region that contacts DrrA (22). To determine if the extreme C terminus of DrrA is the region that makes the above contact with the N-terminal tail of DrrB, cysteine substitutions were created in these two domains. Residue S319 (immediately upstream of the CREEM motif) (Fig. 1.1A) was selected for
the first cysteine substitution in DrrA, and it was tested in conjunction with a substitution S23C within the N-terminal tail of DrrB. This construct was termed A(S319C)B(S23C) to stress the location of the cysteines. (Note that all the cysteine substitution mutants were named as above, unless mentioned otherwise). Strikingly, when membranes containing A(S319C)B(S23C) were treated with homobifunctional (disulfide) cross-linkers, CuPhe (arm length: 0Å) or DTME (arm length: 13.3Å), a species migrating at 65kDa, which was previously identified as the size of DrrA-DrrB heterodimer (22), was detected by anti-DrrA antibody (Fig. 1.2A, lanes 4 and 5). The same species was also detected by anti-DrrB antibody (Fig. 1.2B, lanes 4and5), which implies the formation of DrrA-DrrB heterodimer. These data suggest that the extreme C terminus of DrrA and the N-terminal tail of DrrB are in close proximity and they interact with each other, highlighting the significance of this region of DrrA in mediating the association between the NBD and the TMD of this ABC transporter. To verify the specificity of this interaction, the same cross-linking experiments were carried out with A(S319C)B(C260), which contains the native cysteine at position 260 in DrrB. DrrA-DrrB heterodimer was not formed in this situation, implying that residue 319 in DrrA specifically contacts the N-terminal tail of DrrB (Figs. 1.2A and 1.2B, lanes 6-8).

Two minor species of higher molecular mass (roughly 72kDa, marked with an oblique arrow in Fig. 1.2A) were also identified by anti-DrrA antibody in CuPhe-treated A(S319C)B(S23C) membranes (Fig. 1.2A, lane 4). Since these species were not detected by anti-DrrB antibody, they are likely to be DrrA homodimers, and they correspond in size to the expected size of the DrrA dimer. Interestingly, these potential DrrA homodimeric species become the major cross-linked species in membranes containing A(S319C)B(C260)(Fig. 1.2A, lane 7), when no cysteine is present in the N-terminal tail of DrrB. We have previously shown that DrrA
is trapped in a homodimeric conformation when a cysteine (Y89C) is introduced into the Q-loop of DrrA (23)(also shown in Fig. 1.2A, lane 2, marked with a horizontal arrow). This event reflects the head-to-tail dimerization of the N-terminal catalytic domain of DrrA as well as other ABC proteins (30-32). The DrrA homodimer produced by Y89C-Y89C cross-linking is slightly bigger in size (78 kDa) than the S319C-S319C species, which is likely due to different conformations of the DrrA dimer produced in these two situations. Furthermore, these two dimerization events are likely to be distinct; previous studies showed that Y89C-mediated dimerization of DrrA is affected by ATP (23), whereas S319C-mediated dimerization is not influenced by ATP (data not shown). A species marked as B+B, which corresponds to the size of DrrB homodimer, was also produced in membranes containing either S23C or C260 (Fig 1.2B, lanes 4, 5, 7 and 8). Since this species is produced in all cysteine-containing DrrB variants (22), it is likely to be the result of non-specific association between cysteines in DrrB.

To further define the DrrA-DrrB contact region within the C terminus of DrrA, single cysteine substitutions were introduced both upstream and downstream of residue S319 in DrrA in a strain already containing S23C as the only cysteine in DrrB. Two downstream cysteines introduced within the CREEM motif, A323C and E325C, showed high efficiency of disulfide cross-linking with S23C (Fig. S1.1), as seen previously between S319C and S23C (Fig. 1.2). Analysis of the cysteine substitutions upstream of S319 (at residues 311, 302, 287, 253 or 232) provided different results. In membranes containing A(T311C)B(S23C), two cross-linked species were detected: a minor species of DrrA-DrrB heterodimer identified by both anti-DrrA and anti-DrrB antibodies and a major DrrA dimeric species detected by only anti-DrrA antibody (Figs. 1.3A and 1.3B, lanes 7 and 8). The major species is similar to the 78 kDa DrrA homodimer seen in A(Y89C)B(S23C)(Fig. 1.3A, lane 2). As the location of cysteine substitution moved fur-
ther away from the C-terminal end of DrrA, for example in A(S302C)B(S23C) or A(S287C)B(S23C), DrrA-DrrB heterodimer was undetectable and only DrrA homodimer formation was observed (Figs. 1.3A and 1.3B, lanes 9-14). In A(S253C)B(S23C) or A(S232C)B(S23C), neither DrrA-DrrB heterodimer nor the DrrA homodimer was seen (Figs. 1.3A and 1.3B, lanes 15-20). Taken together, the results discussed above suggest that the extreme C terminus of DrrA participates in two interactions; the region containing the CREEM motif, up to residue S319, is involved in DrrA-DrrB heterodimerization, whereas the 33-residue region upstream of S319 (287 to 319) is involved in DrrA homodimerization. Interestingly, residue 319 is involved in both DrrA-DrrA and DrrA-DrrB interactions, even though the DrrA-DrrB interaction between S319C and S23C is preferred, as seen in Figs. 1.2A and 1.2B.

The C-terminal domain of DrrA contains conserved motifs- To determine if the sequence of the C-terminal domain of DrrA is conserved, last 132 amino acids (residue 199-330), located right after the switch motif, were subjected to NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Surprisingly, this search picked up 99 bacterial and archaeal ABC members. All of these proteins are ABC components of putative doxorubicin or multidrug resistance ABC transporters from various phyla. Even though most of these proteins remain uncharacterized, based on their homology with DrrA it is likely that they belong to the DRA family of ABC proteins. A multiple sequence alignment of all 99 homologs using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) strongly pointed out two highly conserved regions, which we have named LDEADQLA and LDEVFL in this study (Fig. S1.2). The LDEADQLA motif covers 29 residues (199-227), while the LDEVFL motif contains 12 residues (302-313). These motifs are named according to the sequence of the amino acid residues in these motifs, and their location in DrrA is marked in the schematic presented in Fig. 1.1A. Eight
out of the 99 homologs also exhibited a glutamic acid-rich sequence in their extreme C termini, which was defined as CREEM in DrrA in a previous section. These members were, therefore, chosen for a separate multiple sequence alignment using the last 30 amino acid region of these proteins. This alignment highlights both the LDEVFL and the CREEM motifs in this group of proteins (Fig. 1.4A). In summary, we find that the LDEADQLA and LDEVFL motifs are highly conserved in DrrA and all members identified by the Blast search. However, the CREEM motif is conserved in only a few members, which might indicate that CREEM plays a more specific role in function of only some of these transporters.

Interestingly, when a TC-Blast analysis (based on the Transport Classification Database (TCDB, http://www.tcdb.org/progs/blast.php) of the last 132 amino acid sequence of DrrA was performed, it identified mostly eukaryotic homologs, which included ABCA1, ABCA2, ABCA3 and ABCA8 from *Homo sapiens* (and other mammals) and cell death protein 7 (Ced-7) from *Caenorhabditis elegans*. These eukaryotic proteins are evolutionarily more closely related to bacterial proteins, DrrA and NodI, than to the eukaryotic ABC transporters (33) and are assigned to the same family (DRA), but to different subfamilies (DRR and ABCA, respectively) of ABC exporters (9,34). Like most other eukaryotic members of the ABC superfamily, ABCA proteins contain two NBDs and two TMDs within a large molecule (35). DrrA sequence was, therefore, aligned with the corresponding region in NBDI or NBDII of these homologs. It was found that both the NBDI and NBDII of these proteins contain the conserved LDEADQLA and LDEVFL motifs (Figs. S1.3 and S1.4). Only ABCA2 was, in addition, also seen to contain the CREEM motif, which is highlighted by a manual alignment of the last 29 residues of DrrA with the corresponding region of three ABCA2 isoforms from different species (Fig. 1.4B). Taken together, the above analyses allowed the identification of three conserved motifs, LDEADQLA, LDEVFL
and CREEM, within the C-terminal domain of DrrA. Two motifs, LDEVFL and CREEM, at the extreme C terminus of DrrA, were selected for further characterization, as described below. (For clarity, the usage of term ‘extreme C terminus’ in this manuscript refers to the last 50 amino acids and includes both CREEM and LDEVFL motifs of DrrA)

**The conserved motifs in the extreme C terminus of DrrA are involved in function and assembly**- Point mutations and deletions of the conserved residues in the LDEVFL and CREEM motifs were created through site-directed mutagenesis. Mutations created in the LDEVFL motif include L303V, D304N, V306A/F307A/L308A (three residues simultaneously mutated to alanine), L310A/T311A/G312A, and ΔLDEVFL (residues 302-313 were deleted). Mutations in the CREEM motif, as described earlier, include 3E-3G, 3E-3D, 4E-4Q, 5E-5G, and ΔCREEM (residues 321-329 were deleted). Effect of these mutations on expression of the DrrA and DrrB proteins, ATP-binding to DrrA, doxorubicin resistance, as well as doxorubicin efflux was studied. Furthermore, effect of these mutations on interaction between DrrA and DrrB was examined by disulfide cross-linking experiments. Co-purification of DrrA and DrrB on Ni-NTA resin was used as an indicator of assembly of native DrrAB complexes. These studies are described in the sections below.

**Effect of mutations in LDEVFL and CREEM on expression of DrrA and DrrB**- Western blot analysis of the membrane fractions generated from the mutants described above showed that the average DrrA and DrrB expression varied between 80-100% in the different point mutants in the CREEM and LDEVFL motifs (Figs. 1.1B-D; Figs. 1.5A-C), therefore suggesting that these motifs do not play a significant role in stable maintenance of DrrB in the membrane. The strain containing ΔCREEM, however, showed an average reduction of 55% and 78%, respectively, for
DrrA and DrrB (Figs. 1.1B-D), which is most likely due to deletion of multiple residues in this motif.

**Effect on ATP-binding to DrrA:** UV-induced [$\alpha$-$^{32}$P] ATP binding to DrrA was analyzed, as described under Methods. The data in Figs. 1.6A and B, upper gels, represent autoradiograms showing ATP binding, while the lower gels show Western blot analysis of the same blots with anti-DrrA antibodies. The ATP binding efficiency of the wild type DrrA and each mutant was calculated as the ratio of ATP bound (in the presence of doxorubicin) to the amount of DrrA in the sample. The efficiency of ATP binding was then plotted in a histogram designating the efficiency of wild type as 1 (Fig. 1.6C). (Please note that the gels shown in Fig. 1.6A and B are representative, and the data in Fig. 1.6C reflect average of three independent experiments.) Surprisingly, ATP-binding to L303V, V306A/F307A/L308A, L310A/T311A/G312A or \(\Delta LDEVFL\) was found to be abolished both in the absence and presence of doxorubicin (Figs. 1.6A and C), indicating that the residues in the LDEVFL motif are critical for both basal and doxorubicin-stimulated nucleotide binding to DrrA. The D304N mutant was, however, unaffected (Figs. 1.6A and C). In the CREEM motif, 3E-3G, 3E-3D, and 4E-4Q showed normal ATP binding, whereas ATP binding to 5E-5G or \(\Delta CREEM\) was significantly reduced (Figs. 1.6B and C). Together, these results indicate that the conserved motifs in the C terminus of DrrA influence ATP binding in a significant manner.

**Effect on doxorubicin resistance:** Mutations described above were also found to have a significant effect on the overall function of the efflux pump (Table 1.1). It was found that L303V, V306A/F307A/L308A, L310A/T311A/G312A and \(\Delta LDEVFL\) mutations confer doxorubicin sensitivity in the cells, whereas the D304N mutation has only a minor effect on doxorubicin re-
sistance. Mutations in the CREEM motif affected doxorubicin resistance to varying degrees, with 5E-5G and ∆CREEM having the most drastic effect (Table 1.1).

**Effect on doxorubicin efflux**- To further confirm the importance of LDEVFL and CREEM motifs in function, doxorubicin efflux by the DrrAB proteins bearing above mutations was tested. Doxorubicin is fluorescent in solution, however its fluorescence is quenched on binding to DNA inside the cells. This property was used to measure doxorubicin efflux in *E. coli* cells expressing DrrA and DrrB. Efflux was initiated by providing glucose to preloaded *E. coli* cells, and the resulting increase in doxorubicin fluorescence was recorded. The rate of efflux was determined by calculating the slope of the linear portion of each curve shown in Figs. S1.5 A-C. The efficiency of efflux in each LDEVFL or CREEM mutant was then calculated as the percentage of the mutant slope/wild type slope. Control cells containing just the vector showed about 30% efflux of the strain containing wild type DrrAB proteins (Table 1.2). This basal efflux seen in control *E. coli* cells is most likely contributed by the AcrAB pump, which is known to carry out efflux of many different antibiotics by coupling it to the energy of proton gradients (36). Mutations in the LDEVFL motif resulted in varying degrees of effect on doxorubicin efflux. The most severe effect was seen in the L303V, V306A/F307A/L308A and ∆LDEVFL mutants, while D304N remained relatively unaffected (Table 1.2). Of the CREEM mutants, only 5E-5G mutation and ∆CREEM showed a reduction in efflux, while 3E-3G, 3E-3D, 4E-4Q exhibited normal doxorubicin efflux (Fig. S1.5C and Table 1.2; Note that the efflux curves for 3E-3D and 4E-4Q are similar to that of 3E-3G and are not shown in Fig. S1.5C). Overall, the results of the doxorubicin efflux assay are consistent with the doxorubicin resistance results. Taken together, these data show that both LDEVFL and CREEM motifs are essential for the function of the DrrAB transporter. Specifically, residues at positions 303, 306, 307, 308, 310, 311, and 312 in LDEVFL are
key residues that are indispensable for the overall function. Glutamic acid residues in the CREEM motif also play an important role, even though a significant reduction in doxorubicin resistance, doxorubicin efflux, or ATP binding is seen only when all 5 glutamic acid residues are mutated.

**Effect on DrrA-DrrB interaction**- To determine the role of LDEVFL and CREEM motifs in DrrA-DrrB interaction, each of the above mutations was introduced into a strain containing A(S319C)B(S23C). Effect of each mutation on DrrA-DrrB heterodimer formation was studied by disulfide cross-linking between S319C and S23C, as described earlier. The intensity of the DrrA-DrrB cross-linked species produced by CuPhe or DTME was determined by densitometric scanning of the Western blots. The efficiency of cross-linking in each mutant was then calculated as the ratio of the DrrA-DrrB heterodimer (for example, Fig. 1.7A, panel i, lane 7) to the DrrA monomer (from the untreated sample) in the same set (Fig. 1.7A, panel i, lane 6). A similar calculation was done for the wild type sample containing A(S319C)B(S23C) from the same blot (Fig. 1.7A, panel i, lanes 3-5). The efficiency of cross-linking was plotted in a histogram designating the wild type efficiency as 1 (Fig. 1.7A, panel ii). The data in Fig. 1.7A, panels i and ii strongly indicate that the L303V mutation drastically reduces the efficiency of S319C-S23C cross-linking by both CuPhe and DTME. On the other hand, the D304N was found to have no effect on the cross-linking efficiency (Fig. 1.7B, panel i, lanes 6-8 and panel ii). Strikingly, the formation of the DrrA-DrrB heterodimer was completely abolished in V306A/F307A/L308A and ΔLDEVFL mutants (Figs. 1.7C and 1.7E, lanes 6-8). Due to the absence of the detectable DrrA-DrrB heterodimeric species in these two mutants, efficiency of cross-linking could not be determined. (Note that in the strain containing ΔLDEVFL, the size of the DrrA monomer as well as the DrrA homodimer is smaller than the corresponding species in
wild type, as expected) Finally, L310A/T311A/G312A mutant also showed significantly reduced cross-linking efficiency (Fig. 1.7D, panel i, lanes 6-8and panel ii). Together, these data strongly suggest that the LDEVFL motif is crucial for mediating DrrA and DrrB interaction. Once again, residues 303, 306, 307, 308, 310, 311and 312 were found to be essential. Since mutations in these residues do not significantly affect stable expression of DrrA and DrrB, but drastically affect function of the transporter and association of subunits, it can be concluded that interaction between the C terminus of DrrA and the N-terminal tail of DrrB plays a specific role in a higher-order process, such as assembly. In ∆CREEM mutant, the cross-linking efficiency was reduced to between 60-80% of wild type. However, all other CREEM mutations (including 5E-5G) exhibited wild type-like cross-linking between S319C and S23C(data not shown), indicating that the specific interaction between S319 and S23 in DrrB can occur independently.

**Effect on co-purification of DrrA and DrrB** - Co-purification is commonly used as an indicator of association and assembly of protein complexes (37, 38). To confirm the role of the C terminus of DrrA in assembly of the complex, *drrAB* genes bearing the mutations in the LDEVFL or CREEM motif were sub-cloned into pET16b vector, which places a 6xHis tag at the N terminus of DrrA. Co-elution of DrrA and DrrB in the same fraction during the purification process would suggest that these two proteins are associated with each other. The DrrAB complex was solubilized from the membrane fraction with 1% DDM, followed by standard nickel affinity chromatography. The elution profiles of several LDEVFL and CREEM mutants were determined, however only the elution profiles of wild type DrrAB and the LDEVFL mutants, L303V and D304N, are shown in Fig. 1.8A. The data in *lanes 4 and 7, panels* i and ii, show that wild type DrrA and DrrB co-elute in two successive elution fractions, each containing 500 mM imidazole (labeled as fr.1 and fr.2). The L303V mutation, however, drastically affected co-
purification of DrrA with DrrB (Fig. 1.8A, panels i and ii, lanes 5 and 8). The D304N mutation showed only a minor effect (Fig. 1.8A, panels i and ii, lanes 6 and 9). The efficiency of co-purification of DrrA and DrrB was determined by densitometric scanning of the Western blots, and the ratio of DrrB to DrrA purified in each fraction was calculated. Note that the homodimer of DrrB was also observed in both 500 mM fr.1 and 500 mM fr.2, and this species was also scanned and included in the calculation of the amount of DrrB eluted. When plotted in a histogram, the ratio of DrrB to DrrA for the wild type sample was set as 1. The data in Figs. 1.8B and 1.8C show that in addition to the drastic effect of L303V, other LDEVFL mutants, including V306A/F307A/L308A, L310A/T311A/G312A and ∆LDEVFL, also affected the ability of DrrA and DrrB to co-purify. All CREEM mutants tested (including 5E-5G and ∆CREEM) exhibited wild type-like co-purification efficiency (data not shown), showing once again that the region immediately upstream of CREEM (especially residue S319) is able to associate with DrrB independently.

1.4 Discussion

This article describes the identification of two unique motifs, CREEM and LDEVFL, in the extreme C terminus of DrrA that may constitute a novel assembly domain for DrrAB complex in the membrane. Cysteine substitution and cross-linking experiments showed that the amino acid residues S319-E325 (comprising of the CREEM motif and the upstream region up to S319) interact specifically with the N-terminal cytoplasmic tail of DrrB. Interestingly, mutations and deletions created in the LDEVFL and CREEM motifs drastically affected doxorubicin efflux and resistance. Some mutations, in addition, also abolished ATP binding to DrrA. Since these motifs lie far away from the motifs known to be critical for ATP binding to the ABC proteins, a drastic effect of these mutations on ATP binding was unexpected. Moreover, several ABC pro-
teins, including KpsT (*E. coli*) (39) and MJ0796 (*M. jannaschii*) (40,41) do not contain C-terminal extensions, yet they carry out ATP binding successfully. The results obtained in this study can be explained, however, if we consider the fact that ATP binding to DrrA occurs only when DrrA is in complex with DrrB (7). Therefore, if interaction between DrrA and DrrB is disrupted by mutations in either of these motifs, it could affect ATP binding to DrrA. Interestingly, we also found that not all mutations in these motifs compromise ATP binding equally. In some situations, mutations in the neighboring residues resulted in strikingly different effects; for example, the L303V mutation abolished doxorubicin resistance as well as ATP binding, while the D304N mutation showed no effect. Much more extensive mutagenesis analysis is, therefore, needed to better understand the functions of individual residues in these motifs. The most significant finding emerging from the mutagenesis analysis conducted so far is that some mutations in the LDEVFL motif also prevent DrrA and DrrB interaction (judged by disulfide cross-linking) as well as co-purification, which are two indicators of the ability of DrrA and DrrB to form a complex. Therefore, based on the data discussed above, we propose that the CREEM motif of DrrA, including residues up to S319, forms an interaction module for a specific interaction between DrrA and DrrB, while the LDEVFL motif plays a crucial role in regulating this interaction and in assembly of the DrrAB complex. This is a novel, and previously uninvestigated, aspect of ABC protein function and biogenesis. A number of reports have recently appeared in literature suggesting that the C-terminal extensions of ABC proteins may be associated with specialized functions (12-20); that the ABC proteins may play a critical role in membrane protein biogenesis has never been suggested or explored before. Since the LDEVFL and CREEM motifs are conserved in other prokaryotic and eukaryotic homologs belonging to the DRA family, it is likely that these motifs will play a similar role in these homologous systems.
Another interesting aspect of the work presented here is the finding that the C terminus of DrrA also participates in DrrA homodimerization. This function is localized in a 33 amino acid region upstream of S319, and it encompasses the LDEVFL motif. Thus, two interaction interfaces, DrrA:DrrB and DrrA:DrrA, were found to exist in the C-terminal end of DrrA. These studies also indicate that residue S319 exists in equilibrium between A-A ↔ A-B, which raises a number of important questions. For example, under what conditions does the equilibrium shift to the AB species, and vice versa? Further, what is the relationship between DrrA dimerization localized in the C terminus vs. the head-to-tail dimerization of the N-terminal catalytic domain of DrrA, described by us earlier (23). In MalK of *E. coli*, the regulatory C-terminal domains of MalK were shown to remain in contact throughout the catalytic cycle, thus inferring that the C terminus plays a role in stabilization of the MalK dimer (42). Dimerization of the C terminus of DrrA could similarly be involved in stabilization of the DrrA dimer; however we are leaning towards the possibility that this event may be transient and it may be an important prerequisite for DrrA and DrrB interaction. In previous studies using general cross-linkers (such as DTSSP or DSP) DrrA-DrrB heterodimer was the only species detected, while DrrA dimer was never isolated (7), suggesting the transient nature of the DrrA dimer. Furthermore, we find that residue 319 can participate in both A-B and A-A interactions, implying that A-B and A-A species involving the C terminus of DrrA are formed in a mutually exclusive manner.

Several ABC proteins, including MalK, ModC and MetN, which contain regulatory C-terminal domains, have been crystallized recently (17,18,25). Although the C-terminal domains of these proteins do not share significant amino acid identity, they all show the presence of beta-sheet-rich folds characteristic of β-barrel or β-sandwich structures. In ModC and MetN, these domains are critical for binding of the specific pump substrate, resulting in trans-inhibition of
ATPase activity and further substrate uptake (17,18). In the case of MalK, the C-terminal domain binds cytoplasmic proteins MalT and EII<sup>gle</sup>, which plays a role in gene expression and inducer exclusion, respectively (13). We used the closely related bacterial homolog MalK as a template to model the structure of wild type DrrA as well as several mutants in the conserved LDEVFL motif, which are shown through biochemical analysis in this study to negatively influence interaction of the C terminus of DrrA with the N terminus of DrrB and drastically affect function of the DrrAB complex. The model of wild type DrrA, (generated by AMMP modeling software (26)), indicates that the structure of the N-terminal domain of DrrA (containing the ABC cassette) is almost identical to the N terminus of MalK (Fig. 1.9A and 1.9B). Such conservation of structure is not unexpected given the high homology between the N-terminal domains of DrrA and MalK. The C-terminal domain of DrrA exhibits a beta sheet-rich structure which is also similar, but not identical, to the structure of the C-terminal domain of MalK. In the model of DrrA shown in Fig. 1.9B, the LDEVFL motif (shown in yellow) is partially present in beta strand 6, while most of this motif is present in a loop region. Similarly, the CREEM motif (shown in purple) is also seen in a coil or loop region in this model.

Modeling analysis of mutations in the LDEVFL motif, which include L303V, X306-308A, and ΔLDEVFL, showed varying degrees of structural alterations in the C-terminal domain of DrrA (Fig. 1.9C-F). Importantly, however, the structure of the N-terminal domain of DrrA remained largely unaltered in all mutants. Overall, the severity of structural changes observed in the C-terminal domain co-related well with the biochemical effects of mutations reported earlier in this article. For example, D304N mutation which showed minimal effects on DrrAB interaction and function, showed no significant change in the structure of the N- or the C-terminal domain of DrrA (Fig. 1.9C). In the L303V mutant, which significantly affected interaction and
function, beta strands 1 (shown in slate blue) and 4 (shown in orange) present 25 amino acids and 60 amino acids upstream of L303, respectively, were no longer formed (Fig. 1.9D), therefore suggesting that even a conservative mutation in the LDEVFL motif has significant effects on the conformation of the C-terminal domain. Structural changes were also observed in X306-308A mutation; specifically, beta-strands 1 and 6 were not seen. Finally, the most drastic changes, as expected, were observed when the 13 amino acid-long LDEVFL motif was deleted. This deletion resulted in a complete loss of the beta-sheet structure (strands 1-6) of the C-terminal domain, without altering the structure of the N-terminal domain of DrrA (Fig. 1.9F). These analyses not only confirm that the C terminus of DrrA forms an independent domain, but they also indicate that the conserved LDEVFL motif plays a critical role in maintaining the conformational integrity of the C-terminal domain of DrrA, which would be essential for the ability of this domain to interact with the DrrB protein and for overall function of the complex. This conclusion is supported by DrrA and DrrB docking analysis performed by the Rosetta dock server (28), as described under Methods. Results of the docking analysis (shown in Fig. S1.6) indicate that L303V and X306-308A point mutations in the LDEVFL motif significantly alter the orientation of DrrB relative to DrrA. D304N mutation, however, showed no significant effect. Furthermore, the distance between residue S319 in DrrA and S23 in DrrB was also significantly increased in L303V (31.3 Å, Fig. S1.6C) and X306-308A (37.8 Å, S1.6D) as compared to in the wild type (15.5 Å, Fig. S1.6A). In D304N, as expected, the distance (14.3 Å) between these residues remained the same as in the wild type complex (Fig. S1.6B). The modeling analysis of DrrA is therefore consistent with the biochemical studies; both kinds of analyses highlight the importance of the C-terminal domain of DrrA in interaction and function of the DrrAB complex. Further understanding of the roles of the conserved motifs identified in this study will be ob-
tained by genetic analysis, especially by isolation of suppressors of the mutations shown to be important, as well as by crystal structural analysis of the DrrA and DrrB complex.

In summary, we propose that the interaction between the extreme C terminus of DrrA and the N terminus of DrrB, identified in this study, represents an initial interaction important for localization and biogenesis of the complex in the membrane. This is shown as Confo. I in the working model presented in Fig. S1.7, where the N-terminal ATP-binding domains of DrrA are in the ‘open’ conformation, while sequences in the extreme C terminus of DrrA (319-325, including part of CREEM) form an interface with the N-terminal tail of DrrB. It is highly likely that this interaction defines the contact points in the DrrAB heterodimer isolated previously by use of general cross-linkers (7). Confo. II results from binding of doxorubicin to DrrB. In this state the N-terminal tail of DrrB disengages from the C terminus of DrrA. Based on studies reported earlier (23), we suggest that the N-terminal tail of DrrB is now involved in communicating conformational changes to the Q-loop region (represented by residue 89) in the N terminus of DrrA. Simultaneously, the C terminus of DrrA, which is disengaged from DrrB, undergoes homodimerization through the 33-residue region (residues 287-319, including the LDEVFL motif). This is followed by binding of ATP to the N-terminal nucleotide binding domains of DrrA resulting in the ‘closed’ state (confo. III) produced by head-to-tail dimerization of the NBDs (23). Hydrolysis of ATP returns the complex to the resting state (Confo. I). This study defines novel interactions between the ABC component and the transmembrane component of the DrrAB transporter. It raises many interesting questions and opens new avenues for understanding assembly of membrane proteins.

References


**Table 1.1** Effect of mutations in the LDEVFL or CREEM motif on doxorubicin resistance. *E.coli* N43 cells carrying the indicated mutation on a plasmid were streaked on M9 plates containing different concentrations of doxorubicin. The growth was scored after incubation of the plates for 24 h at 37°C. Legend: ++++, very good growth; ++, good growth; +, some growth; +/-: very weak growth; -: no growth. This experiment was repeated four times. Dox, doxorubicin.

<table>
<thead>
<tr>
<th>Motif of DrrAB</th>
<th>mutation</th>
<th>0µg/ml Dox</th>
<th>4µg/ml Dox</th>
<th>6µg/ml Dox</th>
<th>8µg/ml Dox</th>
<th>10µg/ml Dox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td></td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>CREEM</td>
<td>E(321, 322, 325)D</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CREEM</td>
<td>E(321, 322, 325)G</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CREEM</td>
<td>E(321, 322, 325, 326)Q</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CREEM</td>
<td>E(321, 322, 325, 326, 327)G</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CREEM deletion</td>
<td></td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LDEVFL</td>
<td>L303V</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LDEVFL</td>
<td>D304N</td>
<td>+++</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>LDEVFL</td>
<td>V306A/F307A/L308A</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LDEVFL</td>
<td>L310A/T311A/G312A</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LDEVFL deletion</td>
<td></td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 1.2 Effect of mutations in the LDEVFL or the CREEM motif on doxorubicin efflux. Doxorubicin efflux was measured as described under Methods. Based on the data shown in Fig. S1.5, the efficiency of efflux in each LDEVFL or CREEM mutant was calculated, as described in Results. The values shown are the average of the data obtained from three independent experiments. Upper panel shows % efflux in various mutations in the LDEVFL motif. Lower panel shows % efflux in mutations in the CREEM motif. Abbreviations: 3E-3D, E(321, 322, 325)D; 3E-3G, E(321, 322, 325)G; 4E-4Q, E(321, 322, 325, 326)Q; 5E-5G, E(321, 322, 325, 326, 327)G.

<table>
<thead>
<tr>
<th>Efficiency of doxorubicin efflux in DrrA mutants (% of wild type DrrAB efflux)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type Vector L303V D304N V306A/F307A/L308A L310A/T311A/G312A △LDEVFL</td>
</tr>
<tr>
<td>100% 30.6% 31.5% 78% 26.9% 43.5% 35.8%</td>
</tr>
<tr>
<td>Wild-type Vector 3E-3D 3E-3G 4E-4Q 5E-5G △CREEM</td>
</tr>
<tr>
<td>100% 36.5% 100% 97% 109% 54.5% 55%</td>
</tr>
</tbody>
</table>
Fig. 1.1 Schematic representation of the conserved motifs in DrrA. Previously identified motifs, located in the N-terminal domain of DrrA, include Walker A, Q-loop, signature, Walker B, and the switch motif (34). The motifs identified in this study, which include LDEADQLA, LDEVFL and CREEM, are located in the C-terminal domain of DrrA. Numbers indicate the location of specific amino acid residues. The position of residue 319 is also marked. Panel B, effect of mutations in the CREEM motif on expression of DrrA and DrrB. Membranes were prepared as described in Methods. 15 μg membrane protein was analyzed on 12% SDS-polyacrylamide gels, followed by Western blot analysis with anti-DrrA (top gel), anti-DrrB (middle gel), and anti-SecY (bottom gel) antibodies. A non-specific band of about 28 kDa was detected in the anti-DrrB blot in control membranes as well as membranes containing DrrAB proteins. Densitometric scanning of the bands in these blots was carried out as described under Methods. Anti-SecY blot served as a loading control. Panels C and D, histograms showing DrrA and DrrB expression, respectively, in various CREEM mutants. The wild type expression level was designated as 1. The data presented are averages of three independent experiments. Error bars represent standard deviation. Abbreviations used include: 3E-3D, E(321, 322, 325)D; 3E-3G, E(321, 322, 325)G; 4E-4Q, E(321, 322, 325, 326)Q; 5E-5G, E(321, 322, 325, 326, 327)G.
Fig. 1.2 Disulfide cross-linking between S319C in DrrA and S23C or C260 in DrrB. Two different cross linkers, CuPhe (copper phenanthroline) and DTME (dithio-bis-maleimidoethane), were used. Anti-DrrA and anti-DrrB antibodies were used to identify the cross-linked species. Panels A and B, lanes 1, 2: A(Y89C)B(S23C). This strain contains one cysteine in the Q-loop at residue 89 of DrrA and another cysteine in the N-terminal tail of DrrB at residue 23, and it shows the previously characterized DrrA homodimer (23). Lanes 3-5: A(S319C)B(S23C). Lanes 6-8: A(S319C)B(C260). The plus or minus at the bottom of the gels indicates the presence or absence of the cross linker. The position of the two DrrA homodimeric species, present in lanes 4 and 7, is marked with an oblique arrow. This experiment was repeated three times.
Fig. 1.3 Disulfide cross-linking between T311C, S302C, S287C, S253C, or S232C in DrrA and S23C in DrrB. Cysteine scan of the C terminus of DrrA (residues 232-311) was performed by introducing cysteines at indicated positions, followed by disulfide cross-linking assay for each of these cysteine substitution mutants. Panels A and B, lanes 1, 2: A(Y89C)B(S23C); Lanes 3-5: A(S319C)B(S23C); Lanes 6-8: A(T311C)B(S23C); Lanes 9-11: A(S302C)B(S23C); Lanes 12-14: A(S287C)B(S23C); Lanes 15-17: A(S253C)B(S23C); Lanes 18-20: A(S232C)B(S23C). This experiment was repeated three times.
**Fig. 1.4** Sequence alignment of the C terminus of DrrA and its prokaryotic or eukaryotic homologs. *Panel A*, sequence alignment by ClustalW of the last 30 amino acids of DrrA with C-terminal sequences of bacterial homologs identified by NCBI BLAST search. The conserved sequences are termed LDEVFL motif (shown in blue) and CREEM motif (shown in red). *Panel B*, sequence alignment of the last 29 amino acids of DrrA with 25 amino acids of the C terminus of NBDI (upper panel) or 26 amino acids of the C terminus of NBDII (lower panel) of ABCA2 proteins from different species. Both LDEVFL and CREEM motifs are shown by this alignment.
Fig. 1.5 Effect of mutations in the LDEVFL motif on expression of DrrA and DrrB. Conditions described in the legend to Fig. 1.1B were used. Western blot analysis was carried out with anti-DrrA (top gel), anti-DrrB (middle gel), or anti-SecY (bottom gel) antibodies. Anti-SecY blot served as a loading control. Panels B and C, histograms showing the relative amounts of DrrA and DrrB expression, respectively, in various LDEVFL mutants. The wild type expression level was designated as 1. The data presented are averages of three independent experiments. Error bars represent standard deviation. Abbreviations used include: X306-308A, V306A/F307A/L308A; X310-312A, L310A/T311A/G312A.
**Fig. 1.6** Effect of mutations in the LDEVFL or CREEM motif on ATP binding. UV-induced $\alpha^{-32}$P ATP adduct formation was analyzed in membrane fractions, as described under Methods. The reaction was performed both in the presence (marked by +) or absence (marked by -) of 35 $\mu$M doxorubicin. Panels A and B, upper gels, autoradiograms showing $\alpha^{-32}$P ATP binding to wild type DrrAB and various LDEVFL and CREEM mutants. Panels A and B, lower gels, Western blot analysis of the nitrocellulose membranes from the top gels in panels A and B, respectively, using anti-DrrA antibodies. Panel C, a histogram showing the efficiency of ATP binding to wild type DrrA and mutants. The wild type efficiency was designated as 1. The data presented are averages of three independent experiments. Error bars represent standard deviation.
Fig. 1.7 Effect of LDEVFL mutations on disulfide cross-linking between DrrA(S319C) and DrrB(S23C). Each LDEVFL mutation was introduced into a strain containing A(S319C)B(S23C) for disulfide cross-linking experiments. Panel A-i, effect of L303V mutation on disulfide cross-linking. lanes 1-2, A(Y89C)B(S23C); lanes 3-5, A(319C)B(S23C); lanes 6-8, A(S319C)B(S23C) containing the L303V mutation. Western blot analysis was carried out with anti-DrrA antibody. Panel A-ii, a histogram showing the efficiency of cross-linking in wild type and mutant. The wild type efficiency was designated as 1. The data presented are averages of three independent experiments. Error bars represent standard deviation. The column showing DrrA expression indicates the amount of the DrrA monomer. Panels B and D: Effect of D304N and X310-312A(L310A/T311A/G312A) on disulfide cross-linking, respectively. Data are organized as in panel A. Panels C and E: Effect of X306-308A(V306A/F307A/L308A) and ΔLDEVFL on disulfide cross-linking, respectively.
**Fig. 1.8** Effect of mutations in the LDEVFL motif on co-purification of DrrA and DrrB. *Panel A,* Western blot analysis using anti-DrrA and anti-DrrB antibodies. *Lanes* 1-3: Sup, DrrAB proteins solubilized from membrane fraction with 1% DDM; *lanes* 4-6: 500 mM fr. 1, first 500 mM imidazole elution fraction; *lanes* 7-9: 500 mM fr. 2, second 500 mM imidazole elution fraction. The intensity of bands in Western blots was determined by densitometric scanning, and copurification efficiencies were plotted in histograms shown in *panels* B and C. This experiment was repeated three times. Error bars in *panels* B and C represent standard deviation.
**Fig. 1.9** Structure modeling of DrrA. Wild type DrrA and mutants in the LDEVFL motif of DrrA were modeled based on the known crystal structure of MalK (PDB ID:2R6G) using AMMP protein structure modeling software (26), as described under Methods. *Panel A*, crystal structure of MalK (PDB ID:2R6G) is shown in a rectangle. *Panels B-F*, predicted structures of wild type DrrA and mutants in the LDEVFL motif of DrrA. *Panel B*, wild type DrrA; *Panel C*, D304N; *Panel D*, L303V; *Panel E*, X306-308A; *Panel F*, Δ LDEVFL. In all panels, the Walker A, Signature, and Walker B motifs in the N-terminal domain of DrrA are shown in red. The LDEVFL and CREEM motifs in the C-terminal domain are shown in yellow and purple, respectively. The β-strands in the C-terminal domain of DrrA are shown in different colors and are marked as numbers 1-6.
**Fig. S1.1** Disulfide cross-linking between A323C or E325C in DrrA and S23C in DrrB. Two different cross linkers were used: CuPhe and DTME. *Panel A*, anti-DrrA. *Panel B*, anti-DrrB. *Panels A and B*, lanes 1, 2: A(Y89C)B(S23C). *Lanes 3-5*: A(S319C)B(S23C). *Lanes 6-8*: A(A323C)B(S23C); *Lanes 9-11*: A(E325C)B(S23C). The plus or minus indicates the presence or absence of the cross linker.
**Fig. S1.2** ClustalW alignment of the C terminal sequence (residues 199-315) of DrrA with the C-terminal sequences of bacterial homologs identified by BLAST search. Sequences in LDEADQLA and LDEVFL motifs are highlighted.
**Fig. S1.3** ClustalW alignment of the last 132 amino acids of DrrA with the C-terminal sequences of NBDI of eukaryotic homologs identified by TC-Blast search. Their homology in LDEADQLA and LDEVFL motifs are highlighted.
**Fig. S1.4** ClustalW alignment of the last 132 amino acids of DrrA with the C-terminal sequences of NBDII of eukaryotic homologs identified by TC-BLAST search. Their homology in LDEADQLA and LDEVFL motifs are highlighted.
Fig. S1.5 Effect of LDEVFL or CREEM mutations on doxorubicin efflux. *E. coli* LE392ΔuncIC cells containing the indicated plasmids were grown in TEA medium and induced with IPTG at OD=0.6, as described under Methods. Washed cells were de-energized with 5mM DNP and loaded with 10 µM doxorubicin for 11 hours. Loaded cells were washed twice, and doxorubicin fluorescence was measured for 100 seconds. Doxorubicin efflux by the cell suspension was then initiated by providing 20 mM glucose, shown with an arrow. The fluorescence was monitored for an additional 400 seconds. The linear region of each curve was used for calculation of the slope of the curve. Panel A, effect of L303V, D304N or V306A/F307A/L308A mutation on doxorubicin efflux by DrrAB. The slopes obtained were: vector, 82; wild type DrrAB, 331; DrrA(L303V)DrrB, 63; DrrA(D304N)DrrB, 229; DrrA(V306A/F307A/L308A)DrrB, 84. Panel
Fig. S1.6 Docking analysis of the predicted structures of DrrA and DrrB. DrrA protein was modeled using AMMP modeling software and the known structure of MalK as a template. DrrB protein was modeled using the Phyre modeling software (27), as described under Methods. The coordinates obtained for the predicted structures of DrrA and DrrB were then used for docking analysis of DrrA and DrrB by Rosetta Docking server (28). Panel A, wild type DrrA and DrrB; Panel B, D304N and DrrB; Panel C, L303V and DrrB; Panel D, X306-308A and DrrB. In all panels, DrrA is shown in green and DrrB is shown in grey. The Walker A, Signature, and Walker B motifs of DrrA are shown in red. The LDEVFL and CREEM motifs in DrrA are shown in yellow and purple, respectively. The β-strands in the C-terminal domain of DrrA are shown in different colors following the same color scheme as seen in Fig. 1.9. The distances between S319 (shown in magenta) in DrrA and S23 (shown in blue) in DrrB are shown in angstroms.
**Fig. S1.7** A model showing various interactions between DrrA-DrrB and DrrA-DrrA during different stages of the catalytic cycle. Both DrrA and DrrB proteins are shown as dimers. DrrA protein contains two domains: an N-terminal nucleotide binding domain (abbreviated as N-ter, filled with blue) in the front, and a C-terminal domain (showing the CREEM motif) (abbreviated as C-ter) in the front. In Conformation I, the two nucleotide binding domains in the N terminus of the DrrA protein are in the open state, while the extreme C terminus of DrrA forms an interface with the N-terminal tail of DrrB. It is proposed that this interaction between the extreme C terminus of DrrA and the N-terminal cytoplasmic tail of DrrB plays a role in assembly and biogenesis of the DrrAB complex. Doxorubicin binding to DrrB produces a conformational change: the extreme C terminus of DrrA disengages from the N-terminal tail of DrrB, which is now involved in communicating conformational changes to the Q-loop region (represented by residue 89) in the N-terminal domain of DrrA (Confo. II). Simultaneously, the extreme C terminus of DrrA undergoes homodimerization. This is followed by the ‘closed’ state, produced by the head-to-tail dimerization of the NBDs (Confo. III). Hydrolysis of ATP returns the complex to the resting state.
CHAPTER 2
A NOVEL MODULE "GATE" IS ESSENTIAL FOR STABILITY AND ENERGY TRANSDUCTION IN DrrAB COMPLEX

2.1 Introduction

The ATP-binding cassette (ABC) superfamily of proteins is found in organisms from all domains of life. They play pivotal roles in multiple biological processes, including the import of nutrients and export of various toxic molecules and drugs [1]. The efflux of hydrophobic drugs by ABC proteins is implicated as one of the leading causes of multi-drug resistance (MDR) [2]. ABC transporters share a common architecture consisting of two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs). While most eukaryotic ABC proteins contain all four domains within the same polypeptide, the prokaryotic ABC proteins contain one NBD and one TMD fused together in a single polypeptide. In some other cases, the NBD and the TMD are encoded on separate subunits [3]. The NBDs bind and hydrolyze ATP and provide energy for subsequent translocation of substrates through the TMDs.

We are interested in studying the function of a drug efflux system, DrrAB, which confers resistance to the anticancer antibiotics doxorubicin and daunorubicin in the producer organism *Streptomyces peucetius* [4]. This system belongs to the DRA family of ABC proteins to which the eukaryotic proteins of the ABC sub-family A (ABCA) also belong [5]. DrrA, a peripheral membrane protein, plays the role of NBD and forms the catalytic subunit, while the DrrB protein functions as the TMD [6]. Previous studies suggested that DrrA and DrrB together form a tetrameric complex in the membrane [7]. Furthermore, the DrrA and DrrB subunits are biochemically coupled so that proper association between these two proteins is essential for both partners
to achieve stability and active conformation and therefore the overall function of the transporter complex [7, 8].

The N-terminal domain of DrrA contains a 198 amino acid-long ABC cassette consisting of all the conserved motifs that are required for ATP binding and hydrolysis. These motifs include Walker A, Q-loop, Signature motif, Walker B, D-loop, and the Switch motif [9]. A large body of literature has demonstrated the formation of the characteristic head-to-tail interface between NBDs from the opposing ABC subunits within a dimer [10, 11]. In this dimeric state (also referred to as the closed state), the Walker A of one NBD is juxtaposed against the signature motif from the opposing NBD resulting in the formation of two ATP-binding pockets at the interface. Moreover, studies with both DrrA and MalK (ABC subunit of the maltose importer) have shown that the Q-loop region in these proteins facilitates formation of the closed state of the NBDs [9, 12]. Additional data also implied the role of Q-loop in communication of conformational changes between DrrA and DrrB during the catalytic cycle [9].

While the sequence of the N-terminal region of the nucleotide binding domain, which contains the ABC cassette, is highly conserved, the C-terminal domain tends to be highly variable except in closely related ABC proteins. Previous studies on ABC proteins have focused mainly on understanding the function of the ABC cassette which plays a critical role in catalysis and energy transduction in these proteins. Since the C-terminal domain was believed to have only a scaffolding function, its role has remained largely unexplored. Recent studies have shown, however, that these additional domains (when present) may be associated with specialized functions, thus explaining the variability in the sequence of these domains among different ABC proteins. In some ABC proteins, such as ModC of the molybdate/tungstate transporter ModBC in *Methanosarcina acetivorans* and MetN of the methionine transporter MetNI from
*Escherichia coli* (*E. coli*), the C-terminal extension contains a binding site for the pump substrate, which was shown to negatively regulate the uptake activity of the transporter through a trans-inhibition effect [13, 14]. The C-terminal extension of Wzt (the ABC component of the polysaccharide exporter Wzt-Wzm of *E. coli*) also binds the transport substrate; however this function provides substrate specificity rather than trans-inhibition [15, 16]. Additionally, the C-terminal domain of MalK in *E. coli* regulates its activity at both transcriptional and post-translational levels by binding to MalT and IIA\textsubscript{gic}, respectively [17-20]. The osmoregulatory transporter OpuA from *Lactococcus lactis* senses the ionic strength and osmotic stress through its C-terminal extension which possibly forms an alkali ion binding site [21]. The crystal structures of several ABC proteins have become available in recent years. These structures have revealed a common β-sheet fold in their C-terminal domains despite the diversity present in their amino acid sequences, indicating that this structure may be critical for the function of this domain [13-15].

We previously identified three novel motifs in the C-terminal domain (amino acids 199-330) of DrrA [22]. The function of two motifs, LDEVFL (302-SLDEVFLALTGH-313, referred to as DEF in this chapter) and CREEM (321-EEAAEEEEKVA-330) present in the extreme C-terminus of DrrA, was previously characterized in this laboratory [22]. Not only were these motifs shown to be critical for function of the DrrAB complex, but the CREEM motif, together with its upstream region up to Ser319, was also shown to be directly involved in interaction with the N-terminal cytoplasmic tail of DrrB. The DEF motif was found to regulate this interaction and therefore possibly the assembly and function of the DrrAB complex. The DEF motif was also found to participate in homodimerization of DrrA, which may be an important prerequisite for DrrA-DrrB interaction. The 3-dimentional model of DrrA, which was generated using MalK as
a template, showed that the C-terminal domain of DrrA also contains a β-sheet fold structure similar to the one seen in other ABC proteins. Interestingly, the DEF and the CREEM motifs were also found to be conserved among a number of prokaryotic and eukaryotic homologs belonging to the DRA family of ABC proteins, most notable among these being the mammalian ABCA proteins involved in lipid export.

The present study focuses on the third conserved domain, Glycine-loop And Transducer Element (GATE, referred to as LDEADQLA in previous chapter), whose function remains completely unknown. This domain contains 33 residues and is located immediately downstream of the Switch motif of the NBD. The GATE domain is conserved not only among close homologs of the DRA family, but is also found in ABC proteins from other families. We report here the presence of three highly conserved glycines, including G215, G221 and G231, within the GATE domain, which are critical for the structural integrity and function of the DrrAB complex. We found that G215 contacts residues in the Walker A and surrounding area. Therefore it may be indirectly involved in ATP binding and/or catalysis. This residue together with G221 is also shown to be essential for the stability of the DrrAB complex. G231 and nearby residues, including L226 as well as K227, are functionally important and, based on our biochemical data and structural analysis, may constitute a conformational relay between the NBD and TMD during the overall translocation process.

2.2 Material and methods

**Bacterial, plasmids, and antibodies**- The bacterial strains used in this study were *E. coli* TG1, N43, LE392Δunc1C, CC118, and XL1-Blue. The plasmids used included pDx101 (*drrAB* in pSU2718), LA330 (*drrA::lacZ* fusion in pMLB1069), LAB15 (*drrA* and the first 45 base pairs of *drrB::lacZ* fusion in pMLB1069), LAB283 (*drrA-drrB::lacZ* fusion in pMLB1069). Various
substitutions were created in \textit{drrA} and \textit{drrB} genes in the above plasmids. For Western blot analysis, rabbit polyclonal antibodies against DrrA and DrrB proteins were used [6].

\textit{Growth conditions and Media-} For site-directed mutagenesis, XL1-Blue cells were grown at 37°C in NYZ$^+$ broth [pH 7.5, 1% (w/v) casein hydrolysate, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl] supplemented with 12.5 mM MgCl$_2$, 12.5 mM MgSO$_4$ and 0.4% (w/v) glucose (La Jolla, CA). For doxorubicin efflux experiments, cells were grown in TEA medium [50 mM triethanolamine HCl (pH 6.9), 15 mM KCl, 10 mM(NH$_4$)$_2$SO$_4$, 1 mM MgSO$_4$] supplemented with 0.5 % (w/v) glycerol, 2.5 µg/ml thiamine, 0.5 % (w/v) peptone, and 0.15 % (w/v) succinate at 37°C. LB medium was used for all other cell growth unless mentioned otherwise. Chloramphenicol was added to 20 µg/ml, and ampicilin was added to 75 µg/ml, where needed.

\textit{Site-directed mutagenesis-} Both highly and less conservative mutations were introduced into the GATE domain of DrrA by using the QuickChange multi-site-directed mutagenesis kit (Stratagene). For this technique, complementary primers were designed to incorporate the mutation at the desired position. pDx101, LA330, LAB15 and LAB283 were used as templates. Primers were designed as described before [23]. In GATE domain, the highly conservative mutations included E201D, L205V, G215A, G221A, L226V, K227R and G231A. In addition, E201Q, G215S, G215P, G221S, G231S were generated as the less conservative mutations. Cysteine substitutions were constructed in different motifs using the cysteine-less \textit{drrAB} as the template. The cysteine-less \textit{drrAB} was obtained by introducing a mutation C260S in \textit{drrB} in pDX101. The cysteine substitutions included V211C (GATE domain), T222C (GATE domain), S319C (DEF motif) and S23C (N-terminal tail of DrrB).

\textit{Measurement of β-galactosidase activity-} The activity of the fusion proteins was determined by the methods published earlier [23].
**Doxorubicin resistance assay of GATE domain mutants** - The indicated plasmids were transformed into *E.coli* N43 cells. Note that this strain is doxorubicin sensitive. A single colony was inoculated into 5 ml of LB medium supplemented with 20 μg/ml chloramphenicol and incubated at 37 °C for 8h. 1 μl of the cell suspension was streaked onto M9 plates with a top layer containing 0, 4, 6, 8 or 10μg/ml doxorubicin. Since doxorubicin is light sensitive, the plates were covered with foil and incubated at 37 °C for 24 h.

**Doxorubicin efflux assay of GATE domain mutants** - The efflux assay was carried out as described earlier [22]. Briefly, *E. coli* LE392ΔuncIC cells were transformed with the indicated plasmids. Cells were grown in supplemented TEA medium to mid-log phase (optical density 600 nm = 0.6) and induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 1 hour, harvested and washed with TEA twice and resuspended in 1 ml of TEA. 10 μl of the cell suspension from above was incubated in 3 ml of TEA medium containing 10 μM doxorubicin and 5 mM 2, 4-dinitrophenol (DNP) for 11 hours at 37°C. The loaded cells were washed twice with 0.1 M MOPS buffer, pH 7.0, containing 2.0 mM MgSO₄ and resuspended in 3 ml of MOPS buffer. The fluorescence spectra were recorded on an Alphascan-2 spectrofluorometer (Photon Technology International, London, Ontario, Canada). The excitation wavelength for doxorubicin was 480 nm and emission was monitored at 590 nm. The excitation and emission slit widths were set at 0.75 and time based script was run. After an initial recording of fluorescence for 100 seconds at 37°C, energy was provided in the form of 20 mM glucose and recording was continued for an additional 400 s.

The rate of doxorubicin efflux was determined by calculating the slope of the linear portion of each efflux curve. The final slope of each mutant was obtained by subtracting the slope of the control (*E. coli* cells containing the empty vector) from the slope of each efflux curve. The dox-
orubicin efflux efficiency for each GATE domain mutant was then calculated as the percentage of the mutant slope/wild type slope.

**Effect of GATE domain mutants on ATP binding assay** - In order to study the nucleotide binding ability of various GATE domain mutants, pDx101 bearing the indicated mutations were transformed into *E. coli* TG1 cells, which were grown at 37°C to mid-log phase and induced with 0.1 mM IPTG for an additional 3h at the same temperature. The membrane fraction was prepared as follows. Cells were spun down, resuspended in buffer A [25 mM Tris-Cl (pH7.5), 20% glycerol, 2 mM EDTA (pH8.0), 1 mM DTT] and lysed by French press at 16,000 p.s.i.. Cells were centrifuged at 10,000 g at 4°C for 30 min followed by centrifugation of the supernatant at 100,000 g at 4°C for 1h. The obtained pellet was washed twice and finally resuspended in buffer A. The photolabeling of DrrA with [α-32P] ATP was carried out in a 100 µl reaction system containing buffer A, 0.1 mg of membrane protein, 10 µM ATP (pH7.5), 10 µCi of [α-32P]ATP, 35 µM doxorubicin and 5mM MgCl2. The reaction was exposed to UV light at 254 nm on ice for 30 min, followed by protein precipitation by 10% ice-cold TCA on ice for 30 min. Protein was recovered by centrifugation at 14,000 g for 15 min and the pellet was resuspended into 20 µl of 4x Laemmli sample buffer and 5 µl 1M unbuffered Tris. The samples were resolved by polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. The membrane was air-dried and exposed to x-ray film at -70°C overnight, followed by autoradiography. The same blot was then examined by Western blot using anti-DrrA antibody.

**Effect of GATE domain mutants on disulfide cross-linking** - A 100 µl reaction volume containing 250 µg of membrane protein in 0.1M phosphate buffer pH 7.4 (0.1M of Na2HPO4 and 0.1M NaH2PO4) was treated with thio-specific reagents including copper phenanthroline (CuPhe; 3 mM CuSO4, 9 mM 1, 10-phenanthrolne) or 1 mM dithiobismaleimidoethane (DTME, Pierce).
The cross-linking reaction was carried out at room temperature for 30 min and stopped by adding 4x Laemmli sample buffer. A 25 μl portion (50 μg of membrane protein) of the reaction mixture was then analyzed by 10% SDS-polyacrylamide gel, followed by Western blot analysis using anti-DrrA or anti-DrrB antibodies.

**Modeling analysis of GATE domain mutants**- AMMP protein structure modeling software ([24], http://asterix.cs.gsu.edu/ammp.html) was used for homology modeling of DrrA mutants as stated previously [22]. PyMOL Molecular Graphics System ([25], http://www.pymol.org/) was used to measure the atomic distances, view and present protein structures.

### 2.3 Results

**Identification of the GATE domain in DrrA and other ABC proteins**- The N-terminal 198 amino acid nucleotide binding domain of DrrA contains all the conserved motifs involved in ATP binding and hydrolysis [9]. Recent studies from this laboratory identified three novel motifs (CREEM, DEF, and GATE) in the C-terminal 132 amino acid domain of DrrA (residues 199-330). Two of these motifs, CREEM and DEF, present at the extreme C-terminus, were characterized previously [22]. The GATE domain, which is present 104 amino acids upstream of DEF in a region immediately following the Switch motif of the NBD, is the topic of the present study. This domain was found to be highly conserved among close DrrA homologs belonging to the DRA family of ABC proteins (Fig. 2.1A). A multiple sequence alignment of DrrA with ABC transporters outside the DRA family, including MalK, ModC, BtuD, P-glycoprotein (Pgp), Cystic fibrosis transmembrane conductance regulator (CFTR), MsbA, and Sav1866, showed that the GATE domain is also conserved in ABC proteins from other families, albeit with varying degrees of similarity (Fig. 2.1B). The alignments shown in Figs. 2.1A and 2.1B point out that the homology in this region extends across the length of the 33 residue GATE domain. Of these res-
idues, E201, G215, G221, L226, K227, and G231 are the most conserved among DRA family members. Among distant homologs, A206, G215 and G221 are the most highly conserved residues. These observations indicate that G215 and G221 must be critical for the general function of this domain across different ABC families, while the residues conserved only among members of the DRA family may play a more specific role. The crystal structures of several ABC proteins (including MalK, ModC, BtuD, Pgp, CFTR, MsbA and Sav1866) are available [11, 13, 26-29], however no member of the DRA family has been crystallized so far. A comparison of the different crystal structures suggested a high structural conservation in the region of the GATE domain. The structures of MalK, CFTR and Sav1866 shown in Figs. 2.2B-D suggest that this region contains two β-sheets sandwiched by an α-helix on either side. A homology model of DrrA, based on the MalK structure, was developed previously [22], which also showed a very similar fold in the region of the GATE domain (Fig. 2.2A). The highly conserved residue G215 was found to be present in the turn region between the two β-sheets in each structure. This loop is termed the ‘Gly-loop’ (G-loop) in this article. The locations of G215 along with several other conserved residues are marked in Fig. 2.2A and color-coded in Figs. 2.2B-D.

**Conserved glycines (G215 and G221) in the GATE domain are critical for stability of the DrrAB complex**- To determine if the GATE domain is essential for function of the DrrAB complex, site-directed mutagenesis of conserved residues in this domain was carried out resulting in several mutants, including E201D, E201Q, L205V, G215A, G215S, G215P, G221A, G221S, L226V, K227R, G231A and G231S. The Western blot analysis of the membrane fractions generated from the above mutants showed that the expression of DrrA and DrrB was affected to varying degrees in different mutants (Fig. 2.3). For example, mutants E201D, G215A/S/P and G221A/S showed moderate to severe reduction in the levels of DrrA and DrrB expression as
compared to the wild-type, however L205V, L226V, K227R, G231A, and G231S produced no significant effect on expression. Interestingly, when E201 was substituted with glutamine in E201Q mutant, the stable expression of both DrrA and DrrB was almost completely prevented, indicating that the negative charge of E201 may be essential for the stability of DrrAB.

Notably, mutagenesis of the highly conserved residue G215 to G215A, G215S, or G215P completely abolished the expression of DrrB (Fig. 2.3A, lanes 6-8 and Fig. 2.3B). To determine whether the expression was affected at the translational or post-translational level, G215A mutation was introduced into a translational fusion LA330 (constructed previously in this laboratory) created between the extreme C terminus of DrrA (residue 330) and the β-galactosidase gene. The β-galactosidase activity of this fusion served as a measure of the translation level of the *drrA* gene. G231A mutation in DrrA, which showed normal DrrA and DrrB expression on Western blot analysis (Fig. 2.3A, lane 13), was used as a control. The data in Fig. 2.4A show that the translation of DrrA containing the G215A mutation is about 80% as compared to that of wild type DrrA, while the translation of DrrA containing the G231A mutation was unaffected. Similar experiments were also performed with two other translational fusions, LAB15 (in which β-galactosidase is fused to the first 15 amino acids of DrrB) and LAB283 (in which β-galactosidase is fused to the last amino acid, residue 283, of DrrB). The β-galactosidase activity of these fusions served as a measure of the translation levels of the coupled *drr*AB genes. In both the LAB15 and LAB283 backgrounds, the β-galactosidase activity of the strains containing G215A and G231A was comparable to the wild type (Figs. 2.4B and 2.4C), indicating that translation of the *drr*AB genes was not affected by the G215A mutation. Therefore, an additional post-translational effect must be responsible for the observed drastic effect on DrrA and DrrB expression in this mutant. This additional effect may result from the inability of the mutated DrrA
(bearing G215A) to interact with DrrB or from dissociation of DrrA and DrrB due to structural changes (as discussed later). Based on these analyses, we conclude that the G215 residue plays an indispensable role in maintaining the stability and/or proper folding of DrrAB. Furthermore, data in Fig. 2.3 show that the G221A and G221S mutations also significantly affect DrrAB expression though not to the same extent as the G215 mutations. Since either of these glycines could not be changed to Ala or Ser without affecting expression levels in a significant manner, we conclude that both G215 and G221 are critical for maintaining stability of DrrA and DrrB. The third conserved glycine, G231, on the other hand could be mutated to Ala or Ser without affecting expression or stability, indicating that this residue is not involved in stability but may play a specific role in function, as shown later.

**Several other conserved residues (E201, G221, L226, K227, and G231) in the GATE domain are also critical for the function of the DrrAB complex**- To determine if residues in the GATE domain are critical for function, several functional assays, including doxorubicin resistance, doxorubicin efflux and ATP binding, were performed. Doxorubicin resistance analysis showed varying degrees of sensitivity to doxorubicin resulting from different mutations (Table 2.1). To study the effect of mutations on drug transport, de-energized *E. coli* LE392 ΔuncIC cells, containing the mutant DrrA and wild type DrrB, were loaded with doxorubicin, as described under ‘Material and methods’. The DrrAB-mediated doxorubicin efflux in preloaded cells was then initiated by addition of glucose. While fluorescence of doxorubicin is quenched on its binding to DNA inside the cells, its efflux on addition of energy is seen as an increase in its fluorescence (Figs. S2.1- S2.4). The rate of doxorubicin efflux was determined by calculating the slope of the linear portion of each efflux curve shown in Figs. S2.1- S2.4. The final slope of each mutant was obtained by subtracting the slope of the control (*E. coli* cells containing the empty vector) from the
slope of each efflux curve. The doxorubicin efflux efficiency for each GATE domain mutant was then calculated as the percentage of the mutant slope/wild type slope. The data shown in Fig. 2.5 (which represent averages of three independent experiments) suggest that several mutations, including E201D, E201Q, G215A/S/P, G221A/S, L226V, K227R, and G231S, produced a severe reduction in the efficiency of doxorubicin efflux. Of these, E201Q and G215A/S/P contained undetectable levels of DrrB (Fig. 2.3), therefore the actual effect of these mutations on doxorubicin efflux cannot be easily assessed. E201D and G221A/S mutations show moderate (31%-52% of Wild Type) DrrAB expression but are severely affected in doxorubicin efflux (5%-14% of wild type), implying that these residues may be important for the function of the complex. However, the most clear-cut phenotypes were obtained with the L226V, K227R and G231S mutations, which show normal DrrAB expression but a drastic reduction in doxorubicin efflux. Of special note is the difference observed between G231A and G231S mutants. While both show normal DrrAB expression, G231S shows less than 1% doxorubicin “efflux efficiency” but G231A still exhibits about 50% efflux. Since the serine substitution is expected to make the structure of this region more rigid than alanine, these results imply that the residue G231 might be critical for flexibility of this region. However, the flexibility imparted by G231 seems to be more important for function of the complex instead of protein expression or stability. Overall, the efflux data were also found to be largely consistent with the results of the doxorubicin resistance assay shown in Table 2.1, except that L226V mutant showed moderate level of resistance but very low efflux. The reason for this discrepancy is not clear; however, the efflux data are much more quantitative than the resistance data, therefore we believe that the low efflux efficiency accurately reflects the status of this mutant. In summary, the results of these analyses allow us to conclude that the conserved residues E201, G221, L226, K227, and G231 are critical
for the doxorubicin efflux function of the DrrAB transporter. Moreover, the three conserved glycines, G215, G221 and G231, are important for providing structural integrity and flexibility to this region. Of these, G215 and G221 are critical for stability, while G231 plays a crucial role in function.

**Role of the conserved residues of the GATE domain in ATP binding** - To determine if the defect in the doxorubicin resistance and efflux function of certain GATE mutants is due to an effect on ATP-binding, UV-induced [$\alpha$-32P] ATP binding to DrrA was analyzed (Fig. 2.6). The ATP-binding ability of each mutant was normalized by calculating the ratio of ATP bound (in the presence of doxorubicin)/the amount of DrrA in each sample. The efficiency of ATP binding was then calculated as the ratio of ATP binding to mutant/wild type for each mutant, and the averages of the data were plotted in Fig. 2.6B. Even though many mutants were tested for their effect on ATP binding, of special interest were the mutants that showed normal or moderate expression of DrrA and DrrB, but drastically diminished doxorubicin efflux capability, for example E201D, G221A, G221S, L226V, K227R, and G231S (Figs. 2.3 and 2.5). Of these, the K227R mutant, surprisingly, retained full efficiency of ATP binding (Fig. 2.6). Since the efflux function of K227R is significantly compromised, normal ATP binding to this mutant indicates that its function may be compromised in a later stage of catalysis, such as coupling between ATP binding and drug translocation. All other mutants in the category mentioned above retained about 35-57% ATP binding efficiency, but rather poor efflux, therefore it is likely that they are also defective in catalysis (as with K227R). Previous studies have shown that DrrA fails to bind ATP in the absence of DrrB [7]. Therefore, G215 mutations were not tested for ATP binding as no DrrB protein was detected in these mutants. Overall, based on the analyses described above, we
conclude that several highly conserved residues in the GATE domain are critical for the catalytic and the doxorubicin efflux functions of the DrrAB complex.

**The GATE domain does not show direct interaction with the N-terminus of DrrB**- Previous studies from this laboratory showed that DrrA interacts mainly with the N-terminal cytoplasmic tail region (residue 1-53) of DrrB [23]. In chapter 1, we showed that in addition to interacting with the Q-loop region of DrrA, the N-terminus of DrrB forms a dimer interface with the conserved motif CREEM in the extreme C terminus of DrrA [22]. To determine if the GATE domain is also involved in a similar interaction with DrrB, two separate cysteine substitutions, V211C and T222C, were created in a strain containing S23C in the N terminus of DrrB. Homobifunctional cross-linkers CuPhe and DTME were used to treat the membranes generated from the above strains. As shown previously (and in *lanes* 3-5 in Fig. 2.7), when a cysteine is introduced in position 319 which lies immediately upstream of DEF, a strong interaction between S319C in DrrA and S23C in DrrB can be seen with both cross linkers, indicating close proximity between S319 and S23 residues and association between these two regions. However, no DrrAB dimer was seen in the membranes containing V211C(DrrA)/S23C(DrrB) or T222C(DrrA)/S23C(DrrB) (*lanes* 6-11, Figs. 2.7A and 2.7B), which suggests that the GATE domain residues do not interact directly with DrrB.

**Insights from the structural analysis of the GATE domain in MalK and DrrA**- Since the residues of the GATE domain are conserved in many ABC proteins, we performed structural analysis using the crystals of the bacterial ABC protein MalK. The crystal structure of DrrA or the DrrAB complex is so far not available, however the homology model of DrrA, which was published previously [22], was used to obtain additional insights.
Interactions around residue G210 in MalK - The ATP-bound (closed state, PDB access ID: 1Q12) and the ATP-free (open state, PDB access ID: 1Q1E) forms of MalK (*E. coli*), which were published together previously [11], were used for analysis of the conserved residues in the GATE domain. Based on the sequence alignment shown in Fig. 2.1B, the conserved residue G215 in DrrA is equivalent to G210 in MalK. Using the closed state 1Q12, our structural analysis showed that the residue G210 lies in close proximity to the Walker A motif within the same chain of MalK (Fig. 2.8A, shown in cyan color) such that a direct hydrogen bond of N-H...O type with a distance of 2.9 Å from NH group of G210 to carboxylate oxygen of C40 (located within Walker A) was found (Fig. 2.8A). Notably, the Cα of G210 forms another two unconventional hydrogen bonds of the C-H...O type with C40 and V18 at the distance of 3.4 and 3.3 Å, respectively (Fig. 2.8A). It was demonstrated previously that both C40 and V18 in MalK make direct contacts with ATP in the head - to - tail ATP binding interface of the NBD dimer [11]. For example, C40 forms a hydrogen bond with the β phosphate of ATP, whereas V18 stabilizes ATP by van der Waal interaction with the ribose of ATP. Similar atom bonding patterns between G210 and the Walker A residues were also observed in the open state of MalK (1Q1E, shown in slate) though minor variations in distances between atoms were seen (Fig. 2.8A). This observation suggests that the interactions between these residues are maintained stably both in the open and the closed states and probably during the whole ATP catalysis process. No significant interactions were found between other conserved residues (such as those corresponding to G221, L226, K227, G231 in DrrA) in the GATE domain and the Walker A or other regions of MalK.

Superimposition of the open and closed states of MalK - To get a clearer idea of the dynamics of G210 and the Walker A residues during catalysis, 1Q1E and 1Q12 structures were superim-
posed. As seen in Fig. 2.8B, a change from the open state (shown in slate) to the closed state (shown in cyan) results in a movement of C40 (residue shown in white color) by 5.4 Å. A comparable movement of G210 (5.0 Å, residue shown in yellow) and V18 (6.9 Å, not shown) was also observed. This pattern of movement is not limited to these three residues, but is also seen in other important motifs of the NBD, including the complete Walker A loop, the Signature, Walker B, the G-loop region (consisting of A209 and G210) and the two surrounding β-sheets. By comparison, other regions, such as the α-helix immediately after the second β-sheet in the GATE domain, exhibited no obvious movement. These observations imply that ATP binding produces general conformational changes in the regions that are involved in ATP-binding, and these regions may include not only the conventional ATP binding motifs, but also the G-loop in the GATE domain. In summary, G210 forms three hydrogen bonds with residues C40 and V18. These interactions were maintained both in the absence and presence of ATP. Furthermore, superimposition of the ATP bound and ATP-free states of MalK points out that the G-loop moves together with Walker A and other important ATP binding motifs during ATP catalysis.

Insights from the homology model of DrrA and the GATE domain mutants - To get a better understanding of the function of the GATE domain in DrrA, the previously generated 3-dimensional homology model of wild type DrrA was analyzed. The homology models of several mutants, including G215A and K227R, were also generated in the present study using the AMMP software [24]. In the wild type DrrA structure, G215 (which corresponds to G210 in MalK) was once again found to lie in close proximity to A45 (which corresponds to C40 in MalK, [9]) and A23 (corresponding to V18 in MalK) (Fig. 2.8C). However, the distance from the NH group of G215 to carboxylate oxygen of A45 was found to be 3.7 Å, which is longer than required for hydrogen bond formation. Additionally, the distance from the C_A of G215 to the
carboxylate oxygen of A45 and A23 were 4.3 and 3.1 Å, respectively. Thus, an unconventional hydrogen bond C-H...O is formed between G215 and A23 (similar to the bond between G210 and V18 in MalK), however, the other two bonds seen in MalK were not identified in the DrrA model. Even though the homology model is not expected to be as accurate as the crystal structure, modeling analysis allowed a comparison of the structure of wild type DrrA with those of various mutants, thus providing important information in this respect. For example, analysis of the G215A mutant showed dramatic changes in interactions around 215 so that the three distances mentioned above were changed to 2.8 (N_{215}-O_{45}), 3.2 (C_{A215}-O_{45}), and 4.5 Å (C_{A215}-O_{23}), respectively, resulting in a change of between 0.9-1.4 Å in each case (Fig. 2.8D). Analysis of another important residue K227 also provided interesting insights. It was found that K227 forms a C-H...O bond at a distance of 3.5 Å with Ser319 located between the DEF and CREEM motifs in the extreme C terminus of DrrA (Fig. 2.8E). Strikingly, this distance is increased to 12.8 Å in the K227R mutant (Fig. 2.8F). Since S319 is involved in a direct interaction with the N-terminus of DrrB [22], its interaction with K227 could be potentially very important in the function of the GATE domain. The significance of these findings is discussed later.

2.4 Discussion

This study characterizes a recently identified domain, termed GATE, in the ABC protein DrrA [22]. The GATE domain is present in the middle of the DrrA protein with the highly conserved ABC cassette in the N-terminal domain and the conserved DEF and CREEM motifs in the C-terminal domain. Our studies indicate that the GATE domain is conserved in both the close as well as the distant homologs of DrrA (Fig. 2.1), however no biochemical or functional characterization of this domain of the ABC protein family has been reported thus far.
The GATE domain of DrrA is 33 amino acids long and contains three highly conserved glycines, G215, G221, and G231. Here we show that G215 and G221 are mainly involved in maintaining the stability of DrrAB complex, whereas G231 may play an important role in function. Strikingly, mutagenesis of G215 to Ala, Ser, or Pro completely prevented stable expression of DrrB, while mutation of G221 to either Ala or Ser reduced DrrB expression to about 50%. Furthermore, doxorubicin efflux in G221A and G231S mutants was drastically reduced, which indicates that in addition to its role in stability, G221 may also be critical for function of the complex.

β-galactosidase fusion analysis showed that the translation of DrrA and DrrB is unaffected by the G215A mutation in DrrA. Therefore, the effect of G215 mutations on protein expression seems to be post-translational in nature, possibly resulting from the inability of DrrA (containing a mutated G215 residue) to interact with DrrB. It was previously established in this laboratory that the stable expression of DrrB is dependent on its interaction with DrrA expressed either in cis or trans[7]. From the data reported in this chapter, it is evident that the proper conformation provided by the glycines in the GATE domain is an important element for stability of the complex, however the molecular mechanism by which G215 and G221 facilitate DrrAB interaction remains an open question. Whether these glycines play a direct or an indirect role in stability of DrrAB remains to be elucidated. Atomic analysis of the tertiary structure of MalK and the homology model of DrrA showed that the G215 residue forms close contacts with residues in the Walker A motif in the NBD of these proteins. The possible implication of this finding in maintaining stability is discussed later.

Interestingly, mutations of the third glycine G231 did not produce a negative effect on the stability of DrrAB, however a striking difference in the function of the G231A and G231S alleles
was observed. While G231A exhibited normal levels of ATP binding and about 50% doxorubicin efflux efficiency, the G231S mutation reduced ATP binding to about 35% and efflux efficiency to about 1%. Since the serine substitution is expected to make the structure of this region more rigid than alanine, the more drastic effect of the G231S mutation would suggest that the flexibility conferred by G231 is critical for function. Glycines are frequently found in ‘turns’ and ‘bends’ in many proteins, where they are known to confer structural flexibility important for function [30]. The lack of a side chain in a glycine residue allows a domain having glycines the mobility that is necessary for acquiring variability of angles and rotational flexibility. A classical example is the hinge region of the human immunoglobulin G protein (IgG). The mobility of antibody arms in IgG, which is fundamentally important for epitope-IgG interaction, is dependent on the presence of glycine in the hinge region [31]. Further, studies on the DNA-binding proteins such as the lactose repressor (E.coli) and hemagglutinin/protease regulatory protein (Vibrio Cholerae) suggest that Glycines in the hinge region of these proteins are essential for conferring favorable flexibility that is needed for optimal interaction between the protein and target DNA sequences [32, 33]. Our studies, therefore, show for the first time that the conserved glycines in this novel domain of DrrA are important for function of ABC proteins.

In addition to the three conserved glycines, other highly conserved residues in the GATE domain, including E201, L226, and K227, were also found to be critical for the doxorubicin efflux function of the DrrAB transporter. Of special note is the K227R mutation which resulted in significant reduction of doxorubicin efflux efficiency without affecting ATP binding, thus indicating that defective transport in K227R likely results from a defect in a later step in the catalytic process, which is discussed later.
Overall the studies reported in this article suggest that the GATE domain is an interesting and a very important motif for the function of ABC transporters. To gain further insights into its mechanism, we analyzed the crystal structure of the MalK protein since no member of the DRA family has thus far been crystallized. The analysis of the MalK structure at the atomic level revealed that residue G210 (G215 in DrrA), present in the G-loop (Fig. 2.2), forms three hydrogen bonds with residues C40 and V18 in the Walker A motif (Fig. 2.8A), which are known to interact directly with ATP bound at the interface of the MalK dimeric structure [11]. Since these hydrogen bond interactions between G210 and the Walker A residues were seen both in the open and the closed states of the MalK dimer, they likely play a critical role in fixing C40 and V18 in a favorable orientation for ATP-binding. This observation may explain why the G215A mutation in DrrA produces such a drastic effect on the stability of the DrrAB protein complex. Substitution of this Gly with Ala likely abolishes these hydrogen bonds therefore resulting in displacement of these residues and disturbing the structural integrity of the ATP-binding pocket and stability of DrrA. As a consequence, DrrB is expected to be destabilized and degraded since interaction with DrrA is essential for maintaining the stability of DrrB [7]. Therefore, the proper spacing between residue 215 and the residues in Walker A motif may only be accomplished by glycine due to its minimal side chain, which could be essential for integrity of the ATP-binding pocket and, therefore, the stability of the DrrAB complex.

Interestingly, the superimposition of the open and the closed states of MalK showed a comparable and parallel movement of G210 with C40 and V18, suggesting that the G-loop moves together with Walker A and other important ATP binding motifs during catalysis. These findings once again suggest that the constant hydrogen bonding between C40 and V18 in Walker A and G210 in G-loop is essential for producing a favorable conformation required for success-
ful ATP binding. It is very interesting to note that the consensus Walker A sequence (GxxGxGKS/T, where x represents any amino acid) also contains three highly conserved glycine residues, which make hydrogen bonds with the phosphates of ATP [11]. Mutations of these glycine residues in the Walker A of DrrA were previously shown to affect the ATP-binding ability of DrrA as well as the stability of DrrA and DrrB. Therefore, it may be reasonable to propose that along with the three conserved glycines in the Walker A loop, G210 (G215 in DrrA) may also be important for maintaining or constituting the ATP binding pocket.

Analysis of the homology model of DrrA generally supported the findings from the MalK crystal structure in terms of the proximity and interactions between G215 and residues in the Walker A motif, though two of the three hydrogen bonds seen in the crystal structure were not identified in the modeled DrrA structure. This is not surprising since accurate and reliable measurements of atomic distances are not possible with a homology model. Nevertheless, in the absence of any crystal structures of the mutants, modeling analyses allow for comparisons of the structures of the wild type protein with the mutants and can provide important insights. Accordingly, we compared the structure of the wild type DrrA with the G215A as well as the K227R mutant. Interestingly, G215A showed dramatic changes in interactions of G215 with Walker A residues, suggesting that the space around 215 can only accommodate Gly but not Ala. This substitution forced A215 to move towards A45 (C40 in MalK) and away from A23 (V18 in MalK), which may exert a negative effect on structural integrity of the ATP binding pocket, as mentioned earlier. Therefore, based on the structural analysis of MalK and DrrA, we propose that the specific effect of G215 mutations on stability of the complex (especially DrrB) is not due to a direct interaction of the GATE domain with DrrB but occurs indirectly through the effect of these mutations on the integrity of the ATP-binding pocket. This conclusion is also supported by
the cross-linking data shown in Fig. 2.7, where no evidence of disulfide bond formation was seen between V211C or T222C in the GATE domain and S23C in the N-terminus of DrrB. In contrast to V211C or T222C, cysteine substitutions in the extreme C-terminus of DrrA, such as S319C, A323C or E325C, show strong disulfide bond formation with S23C [22], indicating direct interaction between these domains.

Additional important insights were obtained from a comparison of wild type DrrA with the K227R mutant. The drastically affected doxorubicin efflux of K227R indicates that the K227 residue is important for the function of the DrrAB transporter. Interestingly, our structural analysis showed that the K227 residue makes a hydrogen bond (of the C-H…O type) with S319 at a distance of 3.5Å, which is increased to 12.8 Å in the K227R mutant. Since the C terminus of DrrA (which includes residue S319) makes direct contacts with the N terminus of DrrB, as shown in Chapter 1 [22], it is possible that K227 plays a key role in transmitting conformational changes derived from ATP binding to the N-terminus of DrrB through its interaction with S319. Given the fact that the K227R mutant is functionally defective (Fig. 2.5) but shows normal ATP-binding (Fig. 2.6), it is reasonable to propose that ATP catalysis is uncoupled from substrate translocation in this mutant, possibly due to the loss of C-H…O bond between K227 and S319. (Note that the residue K227 is only conserved in the GATE domain of DRA family members, therefore its interacting partners could not be identified in the MalK structure.)

**A proposed model for the role of the conserved motifs in the C-terminal domain of DrrA:**
The biochemical data presented in this article show that the three conserved glycines present in the GATE domain provide structural integrity and flexibility to this region of DrrA, which is critical for maintaining stability and function of the DrrAB complex. The analyses of the crystal structure of MalK and the DrrA model suggest that residue G215 forms hydrogen bonds with
important residues in the Walker A motif which have direct interactions with ATP. We also found that both the Walker A loop and the G-loop (containing G215) move together in a comparable manner on ATP binding. Interestingly, the atomic analysis of amino acid K227, present at the C-terminal side of the GATE domain, showed that it has close interactions with residue S319 present in the extreme C terminus of DrrA. The finding that the mutation of K227 to K227R results in a four-fold increase in its distance from S319, therefore effectively preventing the formation of a hydrogen bond between these residues, is noteworthy. Previous analysis of S319 and the adjoining conserved motifs DEF and CREEM showed that this region is involved in direct interaction with the N-terminus of DrrB, therefore implying the role of K227/S319 interaction in DrrA-DrrB communication.

Putting all of the biochemical and structural analyses together, we propose that the GATE domain functions as a transducer of conformational changes (resulting from ATP binding) from DrrA to DrrB. Specifically, G215 in the G-loop senses the conformational changes resulting from ATP binding to Walker A, which is seen in a comparable movement of the Walker A loop and the G-loop (Fig. 2.8B). K227 in the GATE domain is then able to transduce those changes to DrrB through its interaction with S319. Previous studies from this lab showed that S23 in the N terminus of DrrB also contacts residue Y89 in the conserved Q-loop region of DrrA. Moreover, conformation of the N terminal region of DrrB affects the conformation of Y89C-mediated DrrA homodimer, which led to the proposal that the N terminus of DrrB may be important for transducing conformational changes resulting from doxorubicin binding in DrrB to DrrA [9]. Together, these two sets of studies therefore suggest that the N-terminal cytoplasmic tail of DrrB is involved in 2-way communication between DrrA and DrrB. The first conformational change resulting from doxorubicin binding to DrrB involves transmission from DrrB to the dimeric in-
terface of DrrA, thus causing the closed state of the NBD dimer. The second involves a signal resulting from ATP binding at the dimeric interface of DrrA, which promotes the transition of DrrB from inward-facing to an outward-facing conformation required for drug efflux. In Fig. 2.9, a working model is proposed to illustrate these communications. In Conformation I (Confo. I) of the DrrAB complex, the transport process is initiated by binding of doxorubicin to DrrB. The resulted conformational changes promote the interaction between the N terminus of DrrB (represented by S23C) and Y89C, which in turn brings the two NBDs closer to each other and consequently increases the binding affinity of NBD for ATP. This step explains how ATP binding is stimulated by doxorubicin, a previous finding in our laboratory [6]. Binding of two molecules of ATP produces the fully closed state of the DrrA dimer (Confo. II). The ATP-binding induces conformational changes in Walker A and the G-loop (containing G215), which is then transmitted to DrrB through a K227-S319-S23 cascade. As a result, DrrB undergoes a shift from an inward-facing to an outward-facing conformation, and doxorubicin is translocated out of the cell (Confo. III). Finally, ATP is hydrolyzed, and the complex will be restored to its resting state (Confo. IV). A much more extensive analysis of the conformational changes between different domains of DrrA and DrrB will be carried out in the future studies to elucidate the spatial and temporal sequence of events involved in ATP binding/hydrolysis and the drug transport cycle.

References


Table 2.1 Effect of mutations in the GATE domain on doxorubicin resistance. *E.coli* N43 cells carrying the indicated mutation on pDx101 were streaked on M9 plates containing different concentrations of doxorubicin. The growth was scored after incubation of the plates for 24 h at 37°C. Legend: +++, very good growth; ++, good growth; +, some growth; +/-: very weak growth; -: no growth. This experiment was repeated three times. Dox, doxorubicin.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>0µg/ml Dox</th>
<th>4µg/ml Dox</th>
<th>6µg/ml Dox</th>
<th>8µg/ml Dox</th>
<th>10µg/ml Dox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wild type</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>E201D</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>E201Q</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L205V</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>G215A</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G215S</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G215F</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G221A</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G221S</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L226V</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>K227R</td>
<td>+++</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G231A</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>G231S</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 2.1 ClustalW amino acid sequence alignment of the Glycine Loop And Transducer Element (GATE) domain of DrrA with other ABC transporters. Panel A, alignment with members of the DRA family. Top, alignment with the NBD1 of homologs. Bottom, alignment with the NBD2 of homologs. Panel B, alignment with diverse ABC proteins (both importers and exporters) belonging to different ABC families. The identical residues are highlighted in dark gray, while similar residues are highlighted in light gray. The accession number and source of each protein is as follows: ABCA1 (AAF86276), 2 (AAK14334), 3 (NP_001080) and 8 (AAI30281) are all from Homo sapiens; Pgp (NP_035206) and CFTR (P26361) are both from Mus musculus; Ced-7 (P34358) is from Caenorhabditis elegans; MalK (P68187), BtuD (EGT68374) and MsbA (ABJ00331) are all from Escherichia coli; ModC (2ONK_F) is from Archaeoglobus fulgidus; Sav1866 (Q99T13) is from Staphylococcus aureus. NBD1, nucleotide binding domain 1; NBD2, nucleotide binding domain 2. The PDB accession numbers of the crystal structures of the homologs in panel B are the following. MalK, 2K6G; ModC, 2ONK; BtuD, 1L7V; pgp, 3G60; CFTR, 1R0X; MsbA, 3B5W; Sav1866, 2HYD.
Fig. 2.2 The tertiary structure of the GATE domain of DrrA and other ABC homologs. Panel A, Structure of the GATE domain of DrrA derived from a previously established model of DrrA [22]. Several key residues including A206, G215, G221, and G231 are highlighted in red, yellow, pink and purple colors, respectively, and the equivalent residues in the structures of other homologs (panels B, C and D) are also labeled with the same colors. Panels B-D, Crystal structures of the GATE domains of DrrA homologs. Panel B, MalK; Panel C, CFTR; Panel D, Sav1866. Please refer to Fig. 2.1 for the PDB accession numbers of the crystal structures of the homologs. N, N-terminus; C, C-terminus.
Fig. 2.3 Effect of point mutations in GATE domain on DrrA and DrrB expression. Membranes were prepared as described under ‘Material and methods’. 15 μg of membrane protein was analyzed on 12% SDS-polyacrylamide gels, followed by Western blot analysis with anti-DrrA (top gel) and anti-DrrB (bottom gel) antibodies. A nonspecific band of about 28 kDa was detected in the anti-DrrB blot in control membranes as well as membranes containing DrrAB protein. Densitometric scanning of the bands in these blots was performed by Multi Gauge Version 2.3 (FUJIFILM). Panel B, histograms showing the ratio of mutant/wild type expression using the data obtained by densitometric scanning designated the wild type expression level as 100%. Top, DrrA; bottom, DrrB. Error bars represent standard deviation.
Fig. 2.4 β-galactosidase activity of the \( \text{drrA-lacZ} \) and \( \text{drrAB-lacZ} \) translational fusions. The β-galactosidase activity of the fusion proteins was determined in Miller units per milligrams of total cell protein, as described under ‘Material and Methods’. Panel A, LA330 (\( \text{drrA::lacZ} \) fusion) and LA330-based G215A or G231A mutants; panel B, LAB15 (\( \text{drrA-drrB45nt::lacZ} \) fusion) and LAB15-based G215A or G231A mutants; panel C, LAB283(\( \text{drrA-drrB::lacZ} \) fusion) and LAB283-based G215A or G231A mutants. Panels A-C, β-galactosidase activity of the fusion proteins bearing wild type DrrA or DrrAB was designated as 100%, and the activity of the corresponding mutants was shown as the percentage of the wild type. The data shown represent averages of three independent experiments. Error bars represent standard deviation.
Fig. 2.5 The effect of point mutations in the GATE domain on doxorubicin efflux by the DrrAB transporter. The doxorubicin efflux assay was performed, as described under ‘Material and Methods’. The linear region of each efflux curve (shown in Fig. S2.1-S2.4) was used for calculation of the slope of the curve. The slope exhibited by the host *E. coli* cells (containing the empty vector) was treated as ‘background’ and was subtracted from the calculated slopes of wild type and other mutants. The efficiency of efflux in each GATE domain mutant was then calculated as the percentage of the mutant slope/wild type slope within one set. The data shown here represent an average of three independent experiments. Error bars represent standard deviation.
Fig. 2.6 The effect of the GATE domain mutations on ATP-binding to the DrrAB complex. The UV-induced [$\alpha^{32}$P] ATP adduct formation was analyzed in membrane fractions, as described under ‘Material and Methods’. The reactions were performed either in the absence (marked by -) or presence (marked by +) of 35 µM doxorubicin. The samples were analyzed by 10% SDS-PAGE, followed by transfer to the nitrocellulose membranes. After the membrane was exposed to an X-ray film and developed by autoradiography, the same membrane was probed by anti-DrrA antibodies. Panel A, top, autoradiograms showing [$\alpha^{32}$P] ATP binding to wild type DrrAB and various GATE domain mutants. Panel A, bottom, Western blot analysis of the nitrocellulose membrane from above using anti-DrrA antibodies. Panel B, a histogram showing the efficiency of ATP binding to the GATE domain mutants. The amount of ATP bound was normalized to the amount of DrrA in each sample, as described in “Results”. The ATP-binding efficiency of wild type was designated as 1. The data presented are averages of three independent experiments. Error bars represent standard deviation.
Fig. 2.7 Disulfide cross-linking between cysteines introduced in the GATE domain of DrrA and the N-terminus of DrrB. Two different cross linkers, CuPhe and DTME were used. Anti-DrrA (panel A) and anti-DrrB (panel B) antibodies were used to identify the cross-linked species. Panels A and B, lanes 1-2: A(Y89C). This strain contains one cysteine in the Q-loop at residue 89 of DrrA. The position of the previously identified DrrA homodimer is marked. Lanes 3-5: A(S319C)B(S23C), which bears a cysteine residue in the C-terminus of DrrA and another cysteine in the N-terminus of DrrB. A previously identified DrrAB heterodimer is marked. Lanes 6-8: A(V211C)B(S23C). This strain contains a cysteine at position 211 in the GATE domain. Lanes 9-11: A(T222C)B(S23C), which contains a cysteine at position 222 in the GATE domain. In panel B, a species marked as B+B, which corresponds to the size of DrrB homodimer, was produced in membranes containing either S23C or the native C260. Since this species is produced in all cysteine-containing DrrB variants, it is likely to be the results of nonspecific association between cysteines in DrrB [23]. The plus or minus at the bottom of the gels indicates the presence or absence of the cross linker.
Fig. 2.8 Structural analysis of MalK and DrrA. Panel A, using the crystal structure of MalK, hydrogen bond interactions around G210 in ATP-bound (1Q12) and ATP-free (1Q1E) states of MalK are shown [11]. The carbon backbones of G210 in 1Q12 and 1Q1E are shown in cyan and slate, respectively. Panel B, superimposition of ATP-bound (in cyan) and ATP-free (in slate) forms of MalK. The NBD motifs Walker A, Walker B and ABC signature as well as the GATE domain are shown in both forms. C40 residue is shown in white and G210 is shown in yellow. Panels C-F, using the homology model of wild type DrrA and mutants, atomic distances around critical GATE domain residues are shown. Panel C, G215/A23 and G215/A45 distances in wild type DrrA. Panel D, A215/A23 and A215/A45 distances in G215A mutant. Panel E, hydrogen bond interaction between residues K227 and S319 in wild type DrrA. Panel F, distance between R227 and S319 in K227R mutant. In all panels except panel B, oxygen, nitrogen, and sulfur atom was colored in red, blue, and yellow, respectively. The numbers near dashed lines represent the distances between two atoms.
Fig. 2.9 A model showing 2-way communication between DrrA and DrrB mediated by the N-terminus of DrrB and different motifs in DrrA. Both DrrA and DrrB proteins are shown as dimers. DrrA protein contains two domains: an N-terminal nucleotide binding domain (shown as oval and filled with dark blue), and a C-terminal domain (shown as ⅔ circular shape and filled with light blue). Note that the GATE domain, which belongs to the C-terminal domain of DrrA, is shown as a separate loop in orange. Doxorubicin and ATP are shown as yellow and red, respectively. In conformation I, doxorubicin binds to DrrB which is in its inward-facing conformation. Dox binding produces a conformational change that promotes the communication between the N-terminal tail of DrrB (represented by residue 23) and the Q-loop region of DrrA (represented by residue 89). This interaction transmits the signal of doxorubicin binding to the NBD interface in DrrA resulting in the binding of two molecules of ATP to the binding pocket and the fully closed DrrA head-to-tail dimer (Confor. II). Because of the formation of the closed dimer of the N-terminal domain of DrrA, the C-terminal domains are not completely visible in Confor. II. In the next stage (Confor. III), ATP-binding is communicated from DrrA to DrrB. As described previously, ATP-binding to the NBD triggers conformational changes in the Gly-loop of the GATE domain, which produces a cascade effect finally resulting in S319-S23 interaction. These changes promote transition of DrrB from inward-facing to outward-facing conformation, followed by the extrusion of doxorubicin. In the last step, ATP is hydrolyzed and the complex will be restored to its ATP-free conformation.
Fig. S2.1 Effect of E201D, L205V, G221A or G221S mutation on doxorubicin efflux. *E. coli* LE392ΔuncIC cells containing the indicated plasmids were grown in TEMM medium and induced with IPTG at OD=0.6, as described under Methods. Washed cells were de-energized with 5mM DNP and loaded with 10 µM doxorubicin for 11 hours. Loaded cells were washed twice, and doxorubicin fluorescence was measured for 100 seconds. Doxorubicin efflux by the cell suspension was then initiated by providing 20 mM glucose. The fluorescence was monitored for additional 400 seconds. The linear region of each curve was used for calculation of the slope of the curve. The slope exhibited by the host *E. coli* cells (containing the empty vector) was treated as 'background' and was subtracted from the calculated slopes of wild type and other mutants. The slopes were: wild type DrrAB (green), 822; DrrA(E201D)DrrB (yellow), 167; DrrA(L205V)DrrB (pink), 326; DrrA(G221A)DrrB (blue), 51; DrrA(G221S)DrrB (cyan), 7. The slope of the cells containing empty vector is shown in red color.
**Fig. S2.2** Effect of L226V or K227R mutation on doxorubicin efflux. The slopes were: wild type DrrAB (green), 825; DrrA(L226V)DrrB (yellow), 156; DrrA(K227R)DrrB (pink), 162. The slope of the cells containing empty vector is shown in red color.
**Fig. S2.3** Effect of G231A or G231S mutation on doxorubicin efflux. The slopes were: wild type DrrAB (green), 307; DrrA(G231A)DrrB (yellow), 183; DrrA(G231S)DrrB (pink), 50. The slope of the cells containing empty vector is shown in red color.
Fig. S2.4 Effect of E201Q, G215A, G215S or G215P mutation on doxorubicin efflux. The slopes were: wild type DrrAB (green), 313; DrrA(G215A)DrrB (pink), 31; DrrA(G215S)DrrB (blue), 33; The slope of G215P (cyan) or E201Q (yellow) was lower than the cells containing only vector in this experiment.
GENERAL DISCUSSION

The present studies revealed the existence of two functionally important modules in the C-terminal domain of an ABC protein DrrA. One of the modules present at the extreme C-terminus of DrrA consists of two separate motifs, DEF and CREEM, which are conserved in close homologs belonging to the DRA family of ABC proteins. In the studies described in Chapter 1 [1], it was shown that the CREEM motif (and its immediate upstream region up to S319) interacts with the N-terminus of DrrB and forms an A-B interface. This A-B interaction is significantly impaired by several conservative mutations, such as L303V, in the DEF motif. Unexpectedly, these mutations also deplete the ATP-binding ability of DrrA. It is previously known that DrrA fails to bind to ATP in the absence of DrrB due to its inability to acquire the active conformation by itself [2]. Therefore, it is possible that the DEF mutations result in the improperly assembled DrrAB complex due to the impaired A-B interaction in these mutants. Therefore, the A-B interaction residing in the C-terminus of DrrA is a key factor for proper assembly of DrrAB complex. This conclusion was further supported by the finding that the same set of DEF mutants also drastically affected co-purification of DrrA and DrrB. Based on these biochemical data, we proposed that the A-B association found in this study is regulated by the behavior of the DEF motif; however, the detailed mechanism of this regulation remains to be further studied. Based on the finding that the residues of the DEF motif as well as the upstream region up to amino acid S287 participates in homodimerization of DrrA (which may be a transient interaction), we also proposed that DrrA dimerization may be an important prerequisite for A-B interaction. Additional modeling analysis confirmed that the C-terminus of DrrA forms an independent domain and the DEF motif is critical for conformational integrity of the C-terminal domain of DrrA, which may be essential for A-B interaction [1].
Another distinct functional module, termed GATE, was studied in Chapter 2. This motif is 33 amino acids long and is present immediately after the Switch motif of the NBD in DrrA. Its location between the NBD in the N terminus and the conserved motifs DEF and CREEM in the C terminus suggests that it may play an important role in communication between these domains. Interestingly, the sequence of the GATE domain is highly conserved both among close homologs belonging to the DRA family as well as distantly-related ABC proteins. The most striking sequence conservation is seen in the three glycines, G215, G221, and G231, present in the GATE domain. Different from the DEF and the CREEM motifs (in which the mutants showed normal expression of DrrAB proteins), the significance of this motif was initially recognized by its indispensability in maintaining the stability of DrrAB. While mutations in either G215 or G221 significantly affected stability, the most drastic effect was observed in the highly conservative G215A mutant. This mutant exhibited drastically reduced DrrA and completely depleted expression of DrrB, while the translation of both proteins by lacZ fusion analysis was shown to be normal. Further structural analysis of the crystal structure of MalK [3] provided important insights into the understanding of the role of G215. In MalK, residue G210 (which corresponds to G215) was seen to form hydrogen bonds with two other key residues within or near the Walker A. Since these atomic bonds are maintained in both the ATP-bound and un-bound forms of MalK, it suggests that G210 may be important for maintaining or constituting the ATP binding pocket. Mutagenesis of G215 into Ala may disrupt these bonds and the integrity of the ATP binding pocket, which will produce a negative effect on the stability of DrrA as well as DrrB. Different from G215, mutations of another conserved glycine in the GATE domain, G231, did not have a negative effect on the stability and expression level of DrrAB. However, we found that while G231A exhibited relatively normal levels of ATP binding and doxorubicin
efflux efficiency, the G231S mutation impaired these functional features significantly. Since the serine substitution is expected to make the structure of this region more rigid than glycine or alanine, the drastic effect of the G231S mutation suggests that the structural flexibility conferred by G231 is critical for ATP-binding ability and the overall function of the DrrAB complex. The importance of the glycine residue in conferring the backbone dynamics and conformational flexibility has been emphasized for different categories of proteins [4-7]. Our data reveal that the mutations of the glycine residues in the GATE domain cause two basic types of transporter defects. One type of defect (in the case of G215A) may disrupt the structural integrity of ATP-binding pocket and result in instability of the ABC component and therefore the whole transporter. The other type of defect (in the case of G231S) may disturb the mobility (which may be required for conformational changes essential for ATP catalysis or later stage) of the local region.

Interestingly, our structural analysis also showed that another conserved residue K227 present at the extreme C terminus of the GATE domain forms a hydrogen bond with residue S319 at a distance of 3.5Å. Amino acid S319 is part of the conserved DEF/CREEM module present in the extreme C terminus of DrrA. Strikingly, the distance between K227 and S319 is increased to 12.8 Å in a conservative mutation K227R. The normal expression as well as ATP-binding, but impaired drug transport function of the K227R mutant, promotes the proposal that a later step in the catalytic cycle (such as uncoupling of ATP binding and drug transport) must be disrupted in this mutant. It is therefore possible that K227 plays a key role in transmitting conformational changes derived from ATP binding and/or hydrolysis to DrrB through its interaction with S319.
So far, two DrrA-DrrB interfaces have been identified by studies carried out in our laboratory. These interactions include: (1) Y89 (Q-loop)-S23 (N-terminus of DrrB) [8]; and (2) S319 (C-terminus of DrrA)-S23 [1]. It will be intriguing to understand the roles of these events in communication between DrrA and DrrB during the catalytic cycle and their involvement in energy transduction. Based on the available biochemical and structural data, a catalytic model is proposed in Fig. 2.9 in Chapter 2. In Conformation I, DrrB is in its inward-facing conformation. Doxorubicin binding to DrrB produces a conformational change in the N terminus of DrrB (represented by S23), which then promotes the interaction between S23 and the Q-loop region (represented by Y89) in DrrA [8]. In this step, the signal of doxorubicin binding is transmitted from DrrB to the NBD of DrrA, which increases the affinity of the NBD dimer for ATP. Consequently, two molecules of ATP bind to the ATP-binding pocket, which in turn induces the closed form of the NBD dimer (Conformation II). The next critical step involves transduction of signal of ATP-binding back to DrrB, which is necessary for DrrB to switch from the inward-facing to outward-facing conformation and the extrusion of doxorubicin. ATP-binding induces conformational changes in Walker A and the G-loop (containing G215), which is then transmitted to DrrB through a K227-S319-S23 cascade. As a result, DrrB undergoes a shift from inward-facing to outward-facing conformation, and doxorubicin is translocated out of the cell (Confo. III). Finally, ATP is hydrolyzed, and the complex will be restored to its resting state (Confo. IV). In summary, our studies provide important insights into the associations between the NBD and the TMD of the DrrAB drug transporter and their roles in communicating conformational changes during the catalytic cycle. Future studies will be directed towards in-depth understanding of the detailed mechanisms underlying the G215-K227-S319-S23 signaling cascade and its role in energy transduction.
References


