Mechanistic Insight into β-Lactamase Catalysis, Inhibitor Design and Resistance

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Mechanistic Insight into β-Lactamase Catalysis, Inhibitor Design and Resistance

by

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A dissertation submitted in partial fulfillment of the requirements of the degree of Doctor of Philosophy
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Dedication

To my family.

I never would have gotten here without your support and love.
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List of Abbreviations

PBP ................................................................. penicillin binding protein
ESBL .............................................................. extended spectrum β-lactamase
CRE ............................................................... carbapenem resistant enterobacteriaceae
HB ................................................................. hydrogen bond
LBHB .............................................................. low barrier hydrogen bond
TSA ............................................................... transition state analogue
NMR ............................................................. nuclear magnetic resonance
CDC .............................................................. center for disease control
QM/MM ........................................................ quantum mechanics / molecular mechanics
HTS .............................................................. high throughput screening
RMSD ........................................................... root-mean-squared deviation
LB ................................................................. Luria broth
NAG ............................................................. N-acetylglucosamine
NAM ............................................................. N-acetylmuramic acid
Abstract

The emergence of antibiotic resistance and spread of Gram negative bacteria poses a very real health threat to the public. The main mode of resistance within Gram negative bacteria is the production of β-lactamase enzymes that catalyze the breakdown of β-lactam antibiotics through a hydrolysis mechanism. Once the β-lactam ring is hydrolyzed and opened, the drug loses its efficacy, which allows for the bacteria to grow and proliferate uninhibited. These β-lactamase enzymes are organized into four categories based on the Ambler classification, with classes A, C and D being denoted as serine-based β-lactamase enzymes. Class B is composed of metalloenzymes that use zinc cofactors to catalyze the breakdown of β-lactam antibiotics. The focus of the work presented here today will concern serine β-lactamase catalysis with emphasis on the class A β-lactamase enzymes CTX-M-9, CTX-M-14 and CTX-M-27. This thesis will delve into the underlying mechanics of the class A β-lactamase hydrolysis by way of studying the specific stages of the catalytic mechanism as well as look at nuanced aspects of the active site that leans towards drug discovery and resistance mechanisms. The organization of the document focuses on looking at function analysis, inhibition and resistance of class A β-lactamase.

Understanding the nuances involved in the acid base catalysis of the β-lactamase enzymes will lead to better development of relevant antibiotics that may circumvent resistance mechanisms. With this in mind, I used various techniques can resolve proton locations to help elucidate potential occurrences of low barrier hydrogen bonding exhibited in the mechanism of β-lactamase previously reported during the formation of the pre-covalent complex prior to the acylation transition state. This was done through obtaining a 1.8 Å D₂O exchange apo neutron crystal
structure of CTX-M-9. This was followed up by a perdeuterated structure of CTX-M-9 in complex with the tetrazole scaffold 3GK that was shown to trap the enzyme in the pre-covalent conformation in earlier studies. The resolution of the joint neutron/x-ray structure was 1.7 Å for both neutron and x-ray data sets. The third project entailed using sub angstrom x-ray crystallography to obtain a crystal structure of the deacylation transition state at a resolution of 0.76 Å. The last section of chapter 2 looks at protein engineering, where disulfide bonds are instituted into the active site through mutating residue glutamate 166 to a cysteine. The premise is to selectively engineer perturbations to the microenvironment to help understand the impact on the formation of the low barrier hydrogen bond (LBHB) witnessed between Ser70 and Lys73 in the pre-covalent complex.

The third chapter concerning inhibition of CTX-M-14 and CTX-M-27 focuses on looking at the amide-heteroarene stacking interactions as potential facets for drug design. Different tetrazole-based scaffolds were modified with heteroarene substituents and analyzed for pi stacking interactions with the amide backbone of Gly238. Five crystal structures were obtained and all bound in the predicted fashion with exhibited heteroarene-amide pi stacking interactions, thus lending credence to use CTX-M as a model system.

The last project investigated resistance mechanisms within class A β-lactamase with focus on aspartate – aspartate interactions. Three crystal structures were obtained for this study with the WT CTX-M-14 apo crystal, the CTX-M-14 D233N mutant and the CTX-M-14 D233N J1X tetrazole complex. My study focused on the contribution of aspartate residues to the integrity of the active site with a partial focus on the importance of the 213-219 loop. My findings suggest that β-lactamases can potentially develop resistance against certain inhibitors by increasing its active site flexibility.
Antibacterial Resistance to β-Lactam Antibiotics

The golden era of antibiotics during the 1950s and 1960s ushered in a time of rapid development of many novel compounds used to combat bacterial infections seen in the clinical setting. Comprised primarily of β-lactam containing compounds, these drugs went on to have wide success for a number of decades. β-lactam containing antibiotics work via the inhibition of the formation of the bacterial cell wall and thereby cause cell death through eventual lysis. The mode in which β-lactam binding to penicillin binding proteins (PBPs) is accomplished depends on the resemblance of the β-lactam ring to the D-Ala-D-Ala linkages associated with the terminal pentapeptide linkages of peptidoglycan layers found within the bacterial cell walls, which maintains cell wall rigidity through a cross-linking reaction of the polymeric layer catalyzed by these same DD-transpeptidase enzymes. This cross-linking occurs between two different polysaccharides within peptidoglycan known as N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). The D-Ala-D-Ala linkages are the part of the natural substrate for the PBP enzymes, which get permanently inhibited through the covalent binding of the lactam ring to the PBP active site forming a stable acyl-enzyme complex, and thus render the enzyme ineffective to catalyze the intended cross-linking reaction. The formation of the acyl-enzyme complex does not allow for enzymatic turnover to form cross-linked peptidoglycan polymeric layers and eventually leads to cell death.
Subsequently, resistance to β-lactam antibiotics developed in the clinical setting. The resistance witnessed in bacteria to β-lactam containing drugs can with a large proponent be attributed to the overuse, and misuse, of antibiotics where a large portion of prescribed antibiotics are incorrectly used in cases where they yield little to no medical benefit.\textsuperscript{10-12} Within Gram negative bacteria the main route of resistance is through the hydrolase enzyme known as β-lactamase.\textsuperscript{13,14} Derived evolutionarily from PBPs, β-lactamase enzymes catalyze the breakdown of the β-lactam ring through a hydrolysis mechanism where the lactam is covalently bound to and released by the β-lactamase as an inactive compound, which allows for the bacteria to survive and continue proliferation in the presence of the β-lactam antibiotics.\textsuperscript{15-17} Both β-lactamase and PBP enzymes are efficient at binding β-lactam drugs, but the evolutionary difference lies with the capability of β-lactamase enzymes to undergo a secondary hydrolysis reaction that allows for the release of the hydrolyzed β-lactam compound through a deacylation reaction, thus turning over the enzyme.\textsuperscript{18,19} β-lactamase enzymes are a broad family of enzymes and are commonly referred to by their Ambler classification of A, B, C or D, where classes A, C and D are serine hydrolases, and class B using zinc cofactors to catalyze the breakdown of the lactam ring.\textsuperscript{18,20,21}

**Medical Need for and Importance of β-lactamase Research**

The relative rate of resistant infections has been shown to be increasing at an alarming rate.\textsuperscript{22} The United States Centers for Disease Control (CDC) releases a report that encompasses the most pressing antibacterial resistant infections facing the US that is updated every two to four years. Comparing the numbers of resistant infections from the 2013 report to the 2019 report, the CDC states that there has been a near doubling of the number of deaths witnessed, going from 23,000 in 2013 to 44,000 in 2019.\textsuperscript{22} In 2019 alone, there were over 2.6 million cases of antibiotic resistance reported in clinical settings.\textsuperscript{22} The report encompasses all of the most virulent
microorganisms from with the US and categorizes them as urgent, serious or concerning; where these categories will designate the threat level as well as procedures for dealing with the infections when they occur. Within the body of work presented here, two of these microorganisms will be discussed with focus on carbapenem-resistant Enterobacteriaceae or CRE, which is categorized as an urgent level threat, and extended-spectrum producing β-lactamase or ESBL producing Enterobacteriaceae that is labeled as a serious threat to the public.

CRE bacteria are understandably denoted as an urgent level threat with an approximate mortality rate of 8.4% due to infections. From the 2017 CDC statistics, there were a reported 13,100 cases of carbapenem resistant bacteria in the clinic with a resulting 1,100 deaths. In conjunction with the mortality rate, the estimated costs with associated CRE infections is estimated to 130 million US dollars that includes extended hospital stays, additional trips to the hospital, and cost prohibitive treatment options to deal with the infections that are can be resistant to nearly all available antibiotics approved for use. One aspect of CRE infections that make them difficult to manage clinically is that 30% of these microorganisms carry mobile genetic elements coding for enzymes such as β-lactamase that can be freely shared between bacteria, spreading resistance rapidly in a population.

ESBL producing Enterobacteriaceae are considered a serious threat by the CDC, where the mortality rate associated with these infections is 4.6%. ESBL infections have a higher prevalence compared to CRE with a 197,000 estimated number of cases, with an increase in total ESBL cases from 131,900 that was seen in 2012, with an associated 9,100 deaths reported in 2017; however, ESBL have nearly half the mortality rate of CRE. ESBL infections have an associated economic impact of 1.2 billion that is roughly 10 time higher than CRE infections. Therapeutic options are limited as ESBL are able to hydrolyze penicillin and cephalosporin classes of antibiotics but are able to be treated with carbapenem antibiotics. Unlike some other more opportunistic infections,
ESBL infections are reported to have 25% of the documented cases in people with no preexisting conditions whom are otherwise in good health. Having a better mechanistic understanding of these bacterial infections and modes of antibiotic hydrolysis will help to combat the aggressive infections seen in the clinical setting.

**Ambler Classification and Mechanism of Class A β-lactamase**

β-lactamase enzymes are regularly categorized with the Ambler nomenclature A, B, C and D. For the serine-based hydrolase enzymes from classes A, C and D that use an active site serine to catalyze the binding of β-lactam compounds, they are grouped based on sequence identity and active site composition with the catalytic residues associated with these proteins. In addition, these enzymes can be grouped based upon the substrate profile as well as through ability to perform the hydrolytic mechanism with different modes of action. The emergence of resistance to penicillins and other early precursors to modern β-lactam drugs (Penicillin G, Penicillin F, etc.), drove the development of novel compounds, such as cephalosporins (Figure 1), to combat the resistance seen primarily in classes A and C; however, the overuse and misuse of these compounds led to the appearance of ESBLs. The work presented herein will show resistance profile distinctions that include ESBLs. As the name implies, ESBL enzymes are capable of hydrolyzing a large range of different β-lactam antibiotics including penicillins and cephalosporins and pose a very real clinical threat from the widespread occurrence across the globe.

Class A β-lactamase enzymes are one of the most prolific and widespread classes within the clinical setting that, as a class, are able to hydrolyze virtually all β-lactam drug on the market. The enzymatic mechanism of class A β-lactamases includes high energy intermediate transition states with an acylation and deacylation transition step, both of which involve a proton transfer event that is facilitated by general-acid/base catalysis (Figure 2). Following the
substrate entering the active site, the acylation reaction begins with a water mediated nucleophilic attack of the substrate β-lactam ring by Ser70 after the formation of a high energy alkoxide that primes Ser70, thereby producing a stable covalent acyl-enzyme complex. The second high energy transition state involves a deacylation step, where a water molecule reacts with the acyl-enzyme linkage and facilitates the release of the hydrolyzed product and turnover of the free enzyme. Due to the widespread prevalence of class A β-lactamases found within the clinical setting, the hydrolysis reaction catalyzed by class A β-lactamases has been intensely studied; however, there are many mechanistic and structural details that still remain elusive.\textsuperscript{15, 27, 29, 30} With these questions in mind, the class A β-lactamases model system CTX-M (Cefotaxime Munich) will be used to answer mechanistic questions about protonation states along the reaction coordinate of enzyme turnover, focusing on specific proton transfer events at the high energy intermediate transition states. Previous work with CTX-M-9 and the pseudo wild type system CTX-M-14 have yielded sub angstrom resolution data sets that have helped resolve some of the ambiguity in the formation of the acylation transition state where there has been speculation concerning which residues are important for being the general base compared to the general acid in the activation of the catalytic serine.\textsuperscript{31, 32} Utilizing a 0.86 Å complex crystal structure of CTX-M-14 with a noncovalent inhibitor that mimics the pre-acylation transition state in conjunction with a 0.79 Å crystal structure of the apo system for comparison, this study shows that the water-mediated activation is facilitated via Lys73 directly deprotonating Ser70 as the protonation states are resolved in the unbiased density maps.\textsuperscript{32} Interestingly, there is a phenomenon witnessed in the 0.86 Å complex crystal structure where the hydrogen atom is shared equally between Ser70 and Lys73 at a distance of 2.53 Å.\textsuperscript{32} Such instances are designated as what is known as a low barrier hydrogen bond (LBHB) and are heavily debated but also found in the literature in a wide array of systems (Table 1).\textsuperscript{33}
Low Barrier Hydrogen Bonding

Hydrogen bonds (HBs) play a fundamental role in biological chemistry. Whereas different types of HBs and their properties have been extensively studied in small molecules, the fast development of macromolecular x-ray crystallography in the early 1990s, combined with the use of transition state analogs (TSAs) and NMR, led to the discovery of short HBs in a number of enzymatic systems including, most notably, zinc metallopeptidases, triose-phosphate isomerase (TIM), and serine proteases such as trypsin, chymotrypsin, and subtilisin (Table 1).\textsuperscript{34-36, 37, 38, 39} Compared with standard HBs with a heteroatom distance of 2.8–3.0 Å, these short HBs generally have a length of approximately 2.5 Å. Based on the studies of small molecules, such short HBs are often formed between functional groups with comparable pKa’s, and have a zero point energy of the shared hydrogen higher than the barrier height energy for proton transfer. Thus, these special short HBs were given the name LBHBs by Cleland (Figure 3).\textsuperscript{40} The unique feature of LBHBs results in a delocalized proton equally shared by the two polar heteroatoms in a symmetrical fashion. This is similar to a single-well HB that is even shorter (~2.3 Å) but occurs mostly in some special cases of small molecules. Partially delocalized protons are also observed in asymmetric, short HBs as intermediate types between a standard HB and a symmetrical LBHB. Due to enhanced proton sharing, these asymmetric HBs, like LBHBs, can be stronger than a normal HB. For the discussion herein, such short HBs with partially delocalized hydrogens will also be regarded as LBHBs.

The interest surrounding LBHBs for enzyme studies is due to its extraordinary strength, which can be ~5–10 times stronger than a standard HB in the gas phase.\textsuperscript{41} The initial hypothesis described a transient species at the reaction transition state (TS) when the pKa’s of the donor and acceptor of a HB, involved in general base or acid catalysis, are equal to each other, thus providing an exceptionally strong interaction and serving as the driving force to lower the TS energy.\textsuperscript{41}
Despite the enormous appeal due to its ability to provide a simple answer to the mystery of enzyme catalysis, the concept of LBHBs has been intensely debated from the onset for several reasons. First, the existence of LBHBs in macromolecules was doubted due to the inability to directly observe hydrogen atoms in protein crystal structures in the early days of x-ray crystallography, and there were questions about the presence of these short HBs being potential artifacts of crystal packing. \(^{42}\) Second, the strength of the LBHB was disputed as some studies predicted that LBHBs are much weaker in water and protein microenvironments compared to gas phase calculations, and more on the order of a typical HB. \(^{43, 44}\)

Over the last two decades, the advancement of x-ray and neutron crystallography has elucidated unprecedented details of macromolecular HBs, while new tools of chemical biology have enabled in-depth investigation of individual HBs in protein structure and function. These studies provide important answers about the existence and strengths of LBHBs in the protein microenvironment and reveal potential new roles of LBHBs in protein structures.

**Detection of LBHBs in Protein Systems**

Due to the inherently transient nature of potential LBHBs in enzyme catalysis, and the need to pinpoint the hydrogen atom position, detecting these unique HBs has understandably proven to be a challenge, particularly in the context of protein structures. Small molecule ligands, including transition state analogues (TSAs) and non-covalent inhibitors, have been used to reveal potential LBHBs, while some short HBs have been captured in apo proteins. While NMR and x-ray/neutron crystallography represent the main techniques to analyze LBHBs, infrared and kinetic isotope effects are also useful experimental techniques. \(^{45}\) All of these methods continue to be utilized in the study of LBHBs, but the most progress has been made in the area of crystallography due to the dramatic increase in the quantity and resolution of protein structures. In addition, computational
modeling has become accurate enough to offer unique insights beyond the limitations of experimental techniques.

**LBHB Detection Through NMR**

A staple technique in structural biology, NMR has been used for structural elucidation of more mobile and flexible proteins that are difficult to crystallize for use in x-ray crystallographic studies. 46, 47 Much of the work describing the existence of LBHBs has relied on NMR, with the first early examples in the analysis of chymotrypsin and TIM. 37, 39, 48 LBHBs exhibit a downfield chemical shift for proton spectra of 16–20 ppm, corresponding to a deshielded proton in short HBs. 49 Unlike crystallography, NMR allows the detection of potential LBHBs in a more biologically relevant environment. New examples of potential LBHBs identified by NMR include a HB between Asp32 and the inhibitor hydroxyl group in the complex of endothiapepsin 50, and most recently between His51-Asp75 in the catalytic triad of NS3 serine protease from Dengue virus type II. 51 However, theoretical analysis has suggested that the unique downfield shift can also originate from short ionic HBs where the shared hydrogen is localized. 52 With this understanding and caveat to using NMR for LBHB determination, multiple techniques should be employed for verification of this phenomenon.

**Computational Detection of LBHB: Quantum Mechanical/Molecular Mechanical Calculations**

Computational tools have seen vast improvements over the course of the last few decades with increasing utility at decreased cost to calculate larger, biological systems. 53, 54 With the understanding that LBHB phenomena are quantum mechanical in nature, it is understandable that higher level of theory such as quantum mechanical calculations are needed in order to accurately
reproduce the occurrence using an in silico approach. The issue with using quantum level of theory to calculate biological systems is the associated computational cost with the relatively high electron count within enzymatic systems, which generally use classical-based calculations such as molecular dynamics simulations to tweeze out mechanistic details. In order to be able to calculate larger, more complex systems, a hybrid approach was developed that only treats the area of interest as a higher level of theory, while leaving the rest of the system as classical and was denoted aptly as quantum mechanical/molecular mechanical or QM/MM. This approach has allowed for many complex systems to be calculated with underlying phenomena, such as LBHB interactions, that would be otherwise unable to be accurately calculated.

Prevalence of Short HBs in Protein Structures

Since the LBHB hypothesis was initially formulated, the Protein Data Bank (PDB) has seen its depositions increase from ~500 in 1990, to more than 160,000 to date. Investigation of 1,663 high quality protein crystal structures (<1.1 Å resolution) revealed 3,314 HBs with lengths <2.6 Å and 15,968 HBs with lengths <2.7 Å. These HBs include interactions among both side chain and backbone functional groups, echoing an earlier statistical analysis. Other studies focused on short HBs between pairs of side chain carboxylate groups from aspartate or glutamate residues, found in approximately 14% of protein structures and with an average length of 2.542 Å. Short HBs are also frequently observed in protein-ligand complexes, such as a 2.35 Å HB between Glu52 and the substrate hydroxyl group in a recently determined x-ray complex crystal structure of aminoglycoside nucleotidyl transferase 4′ (ANT4′) from Staphylococcus aureus. Some of these macromolecular HBs, especially between carboxylate pairs, can potentially be LBHBs, while other short HBs, including those involving only protein backbone atoms, do not appear to satisfy the conditions of matching pKa’s for LBHB formation. Some of the short HBs
may partly result from local geometric restraints, including crystal packing artifacts, particularly
under commonly used cryogenic conditions that can cause further shrinkage of crystal volume and
protein structures.\textsuperscript{58,60,61} In addition, positional errors in crystal structures, \textasciitilde0.2 \AA{} for well-ordered
atoms at \textasciitilde2 \AA{} diffraction resolutions, can further complicate the use of the HB length in inferring
its property. For this reason, some early examples of proposed LBHBs, such as the one in
ovotransferrin, may warrant additional scrutiny (Table 1).\textsuperscript{62} Taken together, these observations
suggest a range of short HBs are available in protein crystal structures, and the majority of them
are most likely not LBHBs.

\textbf{Low Barrier Hydrogen Bonding in CTX-M and Enzyme Catalysis}

As mentioned above, previous results from our lab have shown CTX-M-14 class A \(\beta\)-lactamase bound by a non-covalent inhibitor.\textsuperscript{32} The HB between the catalytic Ser70 and the
purported base, Lys73, is 2.53 \AA{} in length, and the hydrogen atom is 1.3 \AA{} from both Ser70O\(\gamma\)
and Lys73N\(\zeta\) in CTX-M-14 class A \(\beta\)-lactamase when bound by a tetrazole-based non-covalent
inhibitor.\textsuperscript{32} Sub angstrom resolution crystal structures of CTX-M-14 class A \(\beta\)-lactamase offer
new clues to potential involvement of LBHBs in enzymatic reactions, especially related to general
base catalysis where proton transfer takes place.\textsuperscript{32} The studies of class A \(\beta\)-lactamase benefit from
a detailed mechanistic understanding of the reaction pathway afforded by QM/MM calculations.
\textsuperscript{27} Hydrolysis of \(\beta\)-lactam compounds proceeds through a covalent acyl-enzyme intermediate
between the catalytic Ser70 and the carbonyl group of the \(\beta\)-lactam ring (Figure 2). Based on
computational predictions, two residues, Glu166 and Lys73, are involved in activating Ser70 in a
concerted base mechanism.\textsuperscript{27} In this model, both Glu166 and Lys73 are charged in the apo enzyme,
but substrate binding triggers a series of proton transfers and converts both residues to a neutral
state. A neutral Lys73 then serves as the general base to deprotonate Ser70. The protonation states
of Lys73 and Glu166 were confirmed by the 0.79 Å resolution apo structure of CTX-M-14 and the 0.89 Å resolution non-covalent complex structure with a tetrazole-based inhibitor.

Interestingly, as previously described, a LBHB was also present between Ser70 and Lys73 in the inhibitor complex, representing the first instance of a LBHB between a nucleophile and the general base where a proton is transferred from the former to the latter during the reaction. In addition, in a third 0.84 Å resolution acylation TSA structure with a boronic acid inhibitor, the potential Ser70-Lys73 LBHB is abolished, and the active site contains a positively charged Lys73 and a neutral Glu166, representing the state immediately after Lys73 deprotonates Ser70. Subsequently, the general acid, Ser130, transfers its proton to the substrate leaving group, the ring nitrogen, while taking a proton from Lys73. This results in a neutral Lys73 and a neutral Glu166 in the acyl-enzyme stage, as captured by another 0.83 Å resolution structure of CTX-M-14 with avibactam.

The above mentioned CTX-M-14 structures have important implications for the investigation of LBHBs, especially related to the use of small molecules. As evidenced by the protonation state change and LBHB formation in the non-covalent complex, ligand binding itself plays an important role in inducing the pKa change necessary for a LBHB between catalytic residues. The absence of the LBHB in the covalent boronic acid complex confirms the common knowledge that TSAs are not perfect mimics of the high energy reaction intermediate and demonstrates that the selection of the right compounds is crucial to capturing specific LBHBs. It is also interesting that QM/MM calculations predicted that general base catalysis takes place at the TS of the acylation reaction, whereas the general acid catalysis occurs during the collapse of the TS. In the acylation TSA structure, a standard HB was observed between the purported general acid, Ser130, and the boronic acid oxygen mimicking the substrate leaving group, the ring nitrogen.
Finally, the strength, or more precisely, the enthalpy of the LBHB, can be crucial to the kinetic process of enzymatic reactions in a pre-organized protein active site, where multiple molecular forces with the necessary strengths have to strike the right locations at specific reaction coordinates. After all, as approximated in the ultra-high resolution x-ray crystal structures, what the shared hydrogen atom has shown is the distortion of electron densities on two functional groups involved in enzyme catalysis. Such details of enzymatic reactions are not captured in the free energy analysis in most of our current experimental approaches. The strength of LBHBs may also be especially important for a proton transfer process where a LBHB has to take place. It is conceptually appealing, and theoretically plausible, to postulate that two functional groups would move closer to form a LBHB to facilitate the proton transfer, as their pKas change along the reaction coordinate. For such LBHBs at least, their unique strength does contribute to lowering the TS energy, because the TS barrier would be higher if the enthalpy of LBHB formation is the same as a standard HB.

Experimental ambiguity remains a key challenge in the study of LBHBs. While crystallography provides the ultimate validation of a delocalized hydrogen, inaccuracy in hydrogen atom positions and crystal artifacts are causes of confusion. Imperfections in perturbation experiments make it difficult to isolate the effect of a single HB. Furthermore, not all experimental techniques are amenable to every protein, leaving gaps in the understanding of a particular macromolecular HB. Compounding these issues is the complexity of enzyme structure and catalysis itself. Multiple short HBs may occur at different stages of the reaction, some at the TS, while others during the formation or collapse of the TS. At a particular reaction coordinate, a network of HBs and other interactions participate in lowering the energy of the reaction intermediate. As the free energy difference between two states is path-independent and is the aggregate result of many competing factors, analyzing the free energy, or enthalpy, of isolated
interactions alone may not capture the full kinetic process of the reaction due to the possibility of undercounting, even when there appears to be an agreement between calculations and some experimental data.

**Fragment-Based Drug Discovery**

Understanding the underlying aspects of the mechanism involving β-lactamase antibiotic hydrolysis will lend itself to a greater impact on how to develop more potent inhibitors against these aggressive bacterial infections. The main approach utilized within our lab focusses on what is known as fragment-based drug discovery, which is a technique that has robust capabilities largely employed by research labs and industry for the last three decades. There is a general workflow associated with this process where researchers will look to designate a viable target that is druggable, design or discover a compound that interacts with the target and then theoretically optimize the potential compound into a high affinity lead compound. The development of a hit compound that interacts with the target can be achieved in a number of ways with commonly used approaches including high-throughput screening (HTS) or molecular modeling. HTS techniques can utilize large ligand libraries with experimental techniques such as surface plasmon resonance to screen for potential hits that have an appreciable interaction between the target and ligand. As with any technique, there are caveats associated with the use of HTS. Namely, the physical screening of ligand libraries is limited by the chemical space of the library itself that may lack the necessary diversity to adequately obtain a high enough affinity hit to be able to move to lead optimization. Also, the technique of using physical screening of proteins and ligand libraries may be cost prohibitive if the target is difficult to purify or the library in question is expensive to use against said target. The premise of using fragment molecules is that being smaller than lead-like compounds with a general molecular weight of less than 300 Da, these compounds are able to
access smaller, more nuanced chemical space on the target protein. Ideally, distinct fragments that are binding to the target protein in sub-pockets clustered near each other, could be tethered together to form a larger, more rigid and higher affinity lead-like molecule from the lower affinity fragment molecules (Figure 4).

**Molecular Docking and Design**

Computational approaches for finding initial hits for drug discovery has become a regularly used tool for many structural biologists and biochemists as the power and speed of calculations increases over time. Molecular docking uses programs to simulate the protein target microenvironment and sample ligand databases that have molecules permutated within the target site with calculated potential binding energies associated between the ligand and target protein. The total energy is generally calculated by what is known as a scoring function, which compartmentalizes the energy of the system in such a way as to facilitate the calculation of the energy of the system in a manner that is as efficient as possible. While there are multiple scoring functions available, the scoring function that will be discussed here is force field-based. These force fields are generally comprised of an energy break down that includes ligand solvation energy, electrostatic interactions, van der Waals interactions and hydrogen bonds with either an explicit solvent or implicit solvent model with a constant dielectric. The main draw that docking programs possess is that, given adequate system parameter choices to reproduce physiological conditions, they will be able to screen millions of compounds quickly without the waste of protein or ligands. In the same vein, biological systems with protein microenvironments are extremely difficult to account for all variables without incurring increased computational cost; therefore, to ensure accurate ligand selection based upon the selected scoring function, an understanding of
what constitutes favorable molecular interactions is needed when inspecting the ligands that are predicted to be top scoring compounds.

**Summary**

Bacteria that express β-lactamase enzymes pose a very real threat to the public, where Gram negative bacteria mainly rely on the use of β-lactamase enzymes to hydrolyze antibiotics for resistance mechanisms. β-lactamase enzymes are organized based on the Ambler classification with the focus of the work presented here being on serine β-lactamase enzymes. The data that will be subsequently organized in the following three chapters will incorporate the analysis of function, inhibition and resistance of class A β-lactamase. Many of the tools outlined in this chapter, such as sub angstrom x-ray crystallography, will be used to investigate the finer mechanistic details concerning the transition states of β-lactamase while also looking at potential low barrier hydrogen bonding interactions. All of the noncovalent inhibitors used in my studies were derived initially from fragment-based drug design and expanded to be used as molecular probes for analysis of β-lactamase catalysis. Understanding the finer points of the acid-base catalysis of β-lactamase would enable the engineering of better, more potent inhibitors.

**Note to Reader #1**

Figure 1. Scaffolds for Classes of β-lactams. From left to right: penicillins, cephalosporins and carbapenems
Figure 2. Catalytic Mechanism of Class A β-lactamase Acylation Mechanism. The binding of the substrate induces protonation state changes in Lys73 and Glu166, either through a series of proton transfers as shown or through a direct proton transfer from Lys73 to Glu166 (stage I). A neutral Lys73 then extracts a proton from Ser70, activating the latter to attack the substrate (stage II). The ensuing TS is stabilized by a LBHB between Ser70 and Lys73, shown by red dashed lines (stage III). During the collapse of the TS, a proton is transferred from Ser130 to the substrate ring nitrogen, followed by a proton transfer from Lys73 to Ser130. This forms the covalent acyl-enzyme intermediate (stage IV). A neutral Lys73 then deprotonates Glu166, enabling the latter to serve as general base and activate the catalytic water to cleave the acyl-enzyme bond. The hydrolyzed product (not shown) is then released from the enzyme active site.
<table>
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<th>Protein</th>
<th>Species/Sources</th>
<th>Residues</th>
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<td>Trypsin</td>
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<td>Asp75-Ala33 in T-S complex</td>
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<td>YjiI</td>
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* TSA, transition state analog.

† Symbol indicates shared hydrogen/deuterium atom identified in the electron/nuclear density map.

‡ Parentheses in Technique, PDB ID, Ref and Year indicate studies against a proposed LBHB.
Figure 3. Energy Potential for a Hydrogen Atom (H) as a Function of its Location Between the Donor (D) and Acceptor (A) Atom. The horizontal line indicates the first vibrational energy of hydrogen. A normal HB has a localized hydrogen, while the hydrogen in a LBHB or single-well HB is completely delocalized. Intermediate types also exist between normal and LBHBs, with partially delocalized hydrogen atoms. The lengths of normal HBs and the intermediate types can be as short as LBHBs, thus HB length alone cannot be used to differentiate different types of HBs.
Figure 4. Scheme Depicting Fragment-Based Drug Discovery. Fragment compounds are docked against designated pockets in the target protein. These compounds are then scored and ranked followed by experimental evaluation. Small, low affinity compounds can be linked together to increase the affinity of the compounds.
Chapter 2:

Functional Analysis of Class A β-lactamase Transition States

Overview

The relative importance of understanding transition states can be directed at the development of more potent inhibitors that specifically target the higher energy conformations of the active site that are formed during β-lactamase turnover. Within this chapter four projects are presented that all target the transition states of CTX-M-9 and CTX-M-14 to some degree, with the first focus on acylation followed by deacylation. The first and second project rely on the use of neutron diffraction experiments with the first structure reported being an apo D$_2$O exchange, proof-of-concept structure. The second structure presented is that of a perdeuterated CTX-M-9 complex with a tetrazole inhibitor that mimics the pre-acylation transition state conformation. These two structures offer direct visualization of the protonation states of the active site of the WT enzyme at different stages of the catalytic cycle. The third project deals with the deacylation transition state that is mimicked using a boronic acid transition state analogue. This 0.76 Å complex of CTX-M-14 is at ultra-high resolution and shows all of the protons in the active site with the unbiased density map. Lastly, the fourth project entails the use of protein engineering in an attempt to establish a method to selectively perturb the active site using a mutation of the catalytic residue Glu166 to a cysteine and induce a disulfide bond to form. I have obtained a series of structures that represent the transition from WT enzyme to mutant structure of Glu166Cys containing an implemented disulfide bond in complex with a tetrazole inhibitor.
Introduction

Transition state analogues have been used as compounds against potential drug targets for a number of years in medicinal chemistry. These high energy states exist in a dynamic, short-lived fashion where conformational changes within the active site or adjacent residues institute the necessary geometry needed to undergo the mechanism. The premise behind using these analogues is that if a particular molecule is able to trap the enzyme in the transition state geometry, which typically only exists for pico or femtoseconds, by mimicking the natural substrate when in the transition state conformation, the protein can adopt a thermodynamically stable state. The design of analogues in this fashion can have inhibitors that bind to the target protein many orders of magnitude stronger than the natural substrate, thus making these analogs ideal targets for drug design and use in probing transition states of different enzymes.

Concerning the mechanism of class A β-lactamase, the question as to whether Lys73 or Glu166 acts as the general base to initiate the priming of Ser70 via formation of an alkoxide in order to bind to the lactam ring was debated within the literature with evidence showing that either residue can act as a general base. Being hydrolase enzymes governed by acid/base catalysis, the mechanism of serine-based β-lactamase enzymes rely on the shuffling of hydrogen atoms, whereby the protonation state of key residues can impact not only ligand binding but also the mechanics of the reaction (Figure 5). Very thorough experimental and computational studies have been performed investigating the protonation states involved with the acylation transition state to elucidate the general base for the initial transition step in the mechanistic pathway. In a QM/MM study conducted by the Mobasherry group, they calculated the energy associated with both paths of the reaction and showed a lower energy associated with utilizing Lys73 as the general base for the initial acylation reaction. The transfer of protons occurs via a route with a negatively charged Glu166 abstracting a proton from a water molecule in the active site that simultaneously
abstracts a hydrogen from Ser70, where the positively charged and adjacent Lys73 donates a hydrogen to Ser70, thus resulting in an overall neutral active site that is representative of a pre-covalent complex. Subsequently, Lys73 abstracts a proton to act as a general base in the formation of the alkoxide Ser70 prior to the nucleophillic attack of the lactam ring.

This chapter will focus on the investigation of different complexes of CTX-M-14 and CTX-M-9 that utilize compounds that mimic the transition states to induce potential LBHB interactions between key catalytic residues involved in the acylation and deacylation transition states. As mentioned in the first chapter, the determination of exact protonation states is a challenge with experimental techniques, which is especially true for transient species with short lifetimes. By nature, LBHB interactions are short-lived species; however, experiments conducted by Nichols et al. concerning binding of a tetrazole scaffold into the active site of CTX-M-14 showed an induced conformational change within the active site microenvironment that enabled the catalytic residues Ser70 and Lys73 to have pKa values altered in such a way as to equally share a proton with a bond distance of 2.53 Å.

**Neutron Diffraction Studies**

Given that LBHB experiments are intensely debated in the literature, unequivocal experimental evidence is needed to help explain such instances when authors predict such phenomena. Comparatively to x-ray diffraction data, neutron diffraction studies allow for direct visualization of the protons within an enzymatic system given the system has undergone D$_2$O exchange for titratable residues or has been perdeuterated (Figure 6). Whereas a hydrogen atom only scatters x-rays weakly due to its lone electron and yields a negative signal in neutron diffraction, deuterium has coherent neutron scattering cross sections comparable to carbon, nitrogen, and oxygen. Thus, given suitable resolutions, neutron diffraction provides a technique
that is better equipped to pinpoint the location of the shared deuterium atom in a potential LBHB. Examples of neutron diffraction experiments have been well documented to show the existence of LBHB within the literature.\textsuperscript{106, 107} The premise for the neutron diffraction and sub angstrom crystallographic studies that are presented in this chapter are derived from the initial study by Nichols et al., which, as previously stated, showed the presence of a LBHB interaction between catalytic residues Ser70 and Lys73 in a pre-covalent complex using sub angstrom resolution x-ray crystallography. This chapter will also show a progression from an apo CTX-M-9 crystal obtained using D\textsubscript{2}O exchange followed by a perdeuterated CTX-M-9 data set, where nonlabile hydrogen have been exchanged for deuterium to obtain a pre-Michaelis complex. The next study will use sub angstrom resolution crystallography to resolve protonation states in the F\textsubscript{o}-F\textsubscript{c} density map.

Results and Discussion

CTX-M-9 D\textsubscript{2}O Exchange Neutron Apo Crystal Structure

The fundamental difference between x-ray diffraction experiments and neutron diffraction experiments is that electron clouds will scatter x-rays, while nuclei will scatter neutrons.\textsuperscript{121} The main caveat to using neutron diffraction studies is the limiting factor of the size of the protein crystal itself. As a proof of concept, an attempt was made to grow CTX-M-9 crystals to the millimeter size scale needed to produce measurable diffraction for structural resolution of the active site protonation states.

Previous structures published with the class A β-lactamase Toho-1 have shown room temperature neutron scattering data collection with the mutations R274N and R276N employed to reduce twinning and increase resolution. The Toho-1 β-lactamase mutants E166A, E166N, or S70A have been used to trap intermediates along the reaction coordinate.\textsuperscript{30} The highest resolution
structures ranged from 1.7 Å at 100K (PDB 5A90) to 1.8 Å at 293K (PDB 6C78). While the mutations of previous structures at residues 274 and 276 were shown not to impact the kinetics of hydrolysis or bonding of smaller lactam containing ligands, focus was placed on obtaining the wild type structure. Thereby, presented here is the isotopically H/D exchanged wild type CTX-M-9 structure at a resolution of 1.85 Å. The reported structure fully reveals the protonation states of the key catalytic residues (Figure 8) in the apo form collected at 293K.

When superimposing the room temperature neutron scattering structure and the previously reported 0.79 Å x-ray structure collected under cryogenic conditions as seen in Figure 9 (PDB 4UA6), there are slight deviations in the active site. While the data presented here support the protonation states of the catalytic residues (i.e., Lys73 and Glu166 both charged) and the hydrogen bonding network observed in the ultra-high resolution x-ray structure, they show a more relaxed active site with the distances between Ser70, Lys73, and Glu166 all deviating by approximately 0.05-0.2 Å. There are two copies of the protein in the asymmetric unit within the P2₁ space group with slight deviations between the two copies. Both copies produce the protonation states of key active site residues, but distances between residues slightly fluctuate. The distances between the functional groups Ser70Oγ and Lys73Nζ varies between 2.44 Å to 3.09 Å for the neutron structure. There is little deviation for the x-ray diffraction structure at 2.75 Å to 2.72 Å between monomeric copies. This larger fluctuation between copies is recapitulated between the other catalytic residues functional groups Glu166Oε₁ and Lys73Nζ. The neutron structure showed distances of 2.99 Å to 2.73 Å in monomers A and B, respectively. The distances within the x-ray structure exhibited no deviation at 2.90 Å between the two copies. Largely this may be because there is more fluctuation within a room temperature crystal structure compared to a structure collected roughly at 77 K, as well as the coordinate errors at the lower resolution of the neutron structure.
Interestingly, in addition to the protonation states of the catalytic residues, there is very well-defined density for residue Cys69 (Figure 8). This is a fairly well conserved residue among class A β-lactamase enzymes and directly abuts the active site residue Ser70, which is involved in binding covalently to substrates. Previous ultra-high resolution x-ray crystal structures deposited for class A enzymes have shown the protonation state of this residue under cryogenic conditions. Typically requiring sub-angstrom resolution to resolve this proton, the structure clearly designates the orientation of the proton on this residue adjacent to the active site. This further confirms the approach of using neutron diffraction studies to resolve residue protonation states and determine the positions of the proton positions, including those that have been difficult to fully interpret from previous x-ray studies alone, even at sub-angstrom resolutions.

Compared with structures determined at cryogenic conditions, room temperature data collection yields a more physiologically relevant active site conformation but is generally prohibitive for sub-angstrom x-ray diffraction due to the radiation damage at synchrotron energy sources necessary to resolve proton positions. Neutron diffraction thus provides a suitable experimental approach for this purpose. This data set establishes this system and protocol for viable neutron scattering experiments.

**CTX-M-9 Perdeuterated Neutron Complex**

The proof-of-concept project outlined with the apo crystal structure of CTX-M-9 demonstrated that it was possible to generate a crystal of sufficient size and quality needed for neutron diffraction experiments. This lead to the generation of a perdeuterated enzyme in order to increase the overall resolution of the system needed to visualize non-labile protons. These efforts resulted in a 1.7 Å joint x-ray/neutron crystal structure that resolves nearly all protons within the system as well as most of the hydrogen positions on the D₂O molecules in the system. The resulting
crystal was on the order of ~1 x 1 x 0.5 mm in total volume, and as such required long periods of ligand soaking to obtain full occupancy. The complex was crystallized in the P2₁ space group that has two copies of the enzyme in the asymmetric unit with R_work/R_free to have values 15.21%/18.23% for the x-ray data with 23.16%/25.55% for the neutron data.

The resolved density in the active site of the CTX-M-9 with 3GK complex structure showed a ligand occupancy of ~90%. When looking at the density of the ligand (Figure 10) there can be seen a distinct difference in the overall density of the ligand when comparing the x-ray and neutron density. The ligand was not deuterated as compared to the enzyme, and as such, the x-ray density to define the presence of the ligand. The x-ray 2 F_o-F_c was contoured at 1.0σ and shows every atom in the ligand. The neutron density 2 F_o-F_c was contoured to 1.5σ and shows most of the main scaffold and highlights the need for joint refinement to get the entire structure resolved. Utilizing the neutron 2 F_o-F_c, we can see direct evidence of the hydrogen bonding with the tetrazole moiety with residues Thr235, Ser237 and Ser130 (Figure 10), which all recapitulate the hydrogen bonding network exhibited by the ultra-high resolution x-ray structure with CTX-M-14 and the same ligand 3GK. For reference, when comparing the systems CTX-M-14 and CTX-M-9, the only difference between the enzymes is a single V231A point mutation. The use of this mutation allows for much higher resolution when performing x-ray diffraction experiments; however, the CTX-M-14 crystals do not grow to adequate size needed for neutron diffraction experiments, hence the use of CTX-M-9 for the presented neutron diffraction data.

When superimposing the 0.86 Å x-ray CTX-M-14 structure (PDB 4UAA) with the 1.7 Å neutron/x-ray structure, minimal deviations within the bond distances of the key catalytic residues and waters in the active site (Figure 11) are seen. The hydrogen bonding network between catalytic residues Ser70, Lys73 and Glu166 and active site waters has been retained with positions deviating ~ 0.1 Å between the cryo x-ray crystal structure and the room temperature neutron/x-ray
These slight bond distance deviations highlight the use of this system as an excellent model to extrapolate comparative findings from the previous sub-angstrom x-ray structure hypothesizing a LBHB interaction between residues Ser70 and Lys73 in a pre-covalent complex.

It has been proposed that a desolvating effect of the active site microenvironment is an important factor in the formation of a LBHB. With the neutron active site retaining nearly the positional characteristics of the comparative x-ray crystal structure 4UAA, it is possible to state that the room temperature neutron data collection has not altered the potential LBHB interaction between Ser70 and Lys73. Similar to 4UAA, the combined neutron/x-ray crystal structure showed solvent occlusion from the active site with the binding of the tetrazole ligand scaffold 3GK as the ligand blocks the active site from bulk solvent with the catalytic triad directly below the inhibitor (Figure 10). It is also worth mentioning that the ligand 3GK will stack upon itself in an antiparallel fashion (not shown in Figure 10 for clarity) that reinforces the occlusion of bulk solvent from the active site.

Comparative bond distances of the key residues in the active site show little distance variations. The distance between atoms on Ser70Oγ and Lys73Nζ shows a minimal change with the distance going from 2.54 Å in the x-ray structure to 2.45 Å in monomer A and 2.55 Å in monomer B within the neutron structure. The slightly shorter bond distance between Ser70 and Lys73 found within monomer A of the neutron structure still falls within the predicted distances associated with LBHB interactions. Looking to residues Ser70Oγ and the oxygen atom in the active site water molecule wat1 there is no change in bond distance at 2.65 Å (Figure 12) between the combined neutron/x-ray structure and the 4UAA structure. The distances from Ser70Oγ to wat2 are 2.63 and 2.51 Å in monomer A and B respectfully and are significantly altered from the distance in 4UAA. Between Glu166Oει and wat1, the bond distances are 2.5 and 2.52 Å when comparing the neutron structure and 4UAA. As can be seen, the bond distances between key
residues have not been appreciably altered and lending credence to a maintained environment that should facilitate a LBHB interaction if judging by the reported spectra defined by 4UAA.\textsuperscript{32}

When looking at the preliminary density map of all the active site residues presented in Figure 13, we are clearly able to see the protonation state of all residues in the active site of the pre-covalent complex are clearly visible, with the exception of residue Ser70. The map is contoured at 1.0 $\sigma$ for the x-ray 2F$_o$-F$_c$ density data and 1.5 $\sigma$ for the neutron 2F$_o$-F$_c$ density data. Using the mechanism provided in Figure 5, it can be predicted that the protonation state of the active site residues should be overall neutral for the pre-covalent complex with Glu166 and Lys73 having gained and lost a proton respectively. The sub angstrom crystal structure showed clearly defined density in the unbiased density map for the protonation states for each of the catalytic triad with a defined density in the middle of Ser70 and Lys73 that has been designated as a LBHB interaction. Residue Glu166 is shown to be neutral (COOH) as predicted by the previous study where Glu166 is acting as a hydrogen bond donor to wat1. While wat1 still acts as a hydrogen bond donor to Asn170, the deviation concerning wat1 in the combined neutron/x-ray structure compared to 4UAA, is the lack of a hydrogen bond being directed at Ser70. Interestingly, in the neutron density shown in Figure 13, Lys73 is shown to be neutral (NH$_2$); however, there is no density between Lys73 and Ser70. The location of the hydrogen on Ser70 is somewhat ambiguous as it could oscillate between Lys73 or the nearby catalytic water wat1 in a donor fashion. When referencing the CTX-M-14 structure previously published, it needs to be taken into consideration that the structure was collected under cryogenic conditions with the comparative difference between 77 K and 297 K potentially having a large impact on the relative placement of the hydrogen on Ser70. When looking to the seminal, groundbreaking work by Cleland, where he hypothesized that LBHB interactions are transient and difficult to isolate experimentally, we can see the forethought where he implied that LBHBs could theoretically be implemented to stabilize activated serine residues
prior to nucleophilic attack on a substate. The discrepancy between the cryo sub angstrom x-ray data of CTX-M-14 and the room temperature neutron data set of CTX-M-9 needs to be addressed by potentially performing the same experiment at cryo conditions. Ideally, as all of the bond distances of key catalytic residues has been maintained and since both Lys73 and Glu166 are clearly defined with density for neutral protonation states where Lys73 is acting as a hydrogen bond acceptor with Ser70, performing a neutron diffraction experiment at 77 K might help stabilize the hydrogen on Ser70. Regardless of the ambiguous protonation state of Ser70, the room temperature neutron data set presented here does not contradict previous reported data for this system and supports the predicted protonation states of the catalytic triad congruent with the pre-covalent complex.

**Deacylation Transition State and Sub Angstrom Crystallography**

In the same vein as trying to use crystallographic techniques to visualize the protonation states as well as hydrogen locations for the pre-acylation complex, an attempted was made to elucidate the protonation state of the deacylation transition state for the class A β-lactamase mechanism. Previous studies used cephalosporin and penicillin mimic compounds as a starting block to attempt to trap the target enzyme in a tetrahedral intermediate that structurally resembled the acylation or deacylation transition state. Such example compounds, like CB4 and SM23, have been used to generate high resolution crystal structures that showcase the mechanistic pathway of class A β-lactamase along the reaction coordinate for the acylation or deacylation transition states respectively. These boronic transition state analogues work in a similar route as that of penicillins or cephalosporins, but unlike the β lactams, the glycyloboronic acids were designed to have a reversible competitive binding mode of action where, when bound, they are structurally analogous to the quaternary structure of β-lactam hydrolysis. The reversible
reaction forms fast-on as well as fast-off lewis acid adducts between the glycyloboronic acids and serine based β-lactamase enzymes.\textsuperscript{124} With this in mind, compound SM23 was used in an attempt to try and resolve a sub angstrom resolution crystal with CTX-M-14 to elucidate the protonation state of the catalytic residues during the deacylation transition state.

The resulting crystal structure that utilized the boronic acid transition stat analogue SM23 was obtained at a resolution of 0.76 Å and resolves nearly all the protons in the protein. The enzyme was crystallized in the \textit{P2}_1 space group with two copies in the assymetric unit. The preliminary statistics shows R\textsubscript{work} and R\textsubscript{free} values of 12.88\% and 13.72\% respectively. The boronic acid transition state analogue ligand SM23 (Figure 14) is shown in clear detail in both \textit{F}_o-\textit{F}_c and 2\textit{F}_o-\textit{F}_c maps where two conformations of the thiophene ring can be seen (Figure 15). The second copy of the thiophene ring induces Ser237 to be rotated away from the active site to avoid steric clashes. The ligand is near 99\% occupancy in the active site and completely displaces the phosphate ion that is typically situated between Lys234 and Ser237 within the apo crystal structure.

Looking at the density of the residues in the active site, we can see atomistic detail within the 2\textit{F}_o-\textit{F}_c biased density map and even some of the non-polar hydrogen atoms are resolved (Figure 16). Utilizing the unbiased \textit{F}_o-\textit{F}_c density map, it is possible to see all catalytically relevent protons in the active site, thus clearly defining the protonation state of the deacylation transition state. Lys73 is positively charged, exhibiting hydrogen bonds between Ser70\textit{O}_{γ} and Lys73N\textit{ζ} (distance of 2.7 Å) and between Lys73N\textit{ζ} and Asn132\textit{O}_{δ1} (distance 2.67 Å). Glu166 is neutral and hydrogen bonding to the boronyl oxygen atom. Looking to the mechanism of class A β-lactamase enzymes in Figure 5, it can be seen that the experimental protonation states match well with the predicted states.

A particularly hydrogen bond is present between Glu166\textit{O}_{ε1} and the boronyl oxygen (distance 2.47 Å) (Figure 17). When looking at the unbiased \textit{F}_o-\textit{F}_c density map, there is density in
the middle of the bond that is partially localized closer to the Glu166Oε1. Fully protonated carboxylic acids have unequal bond distances between the terminal oxygen atoms and connecting carbon on the order of 1.21 Å for the carbonyl and 1.30 Å for the C-OH hydroxyl bond distance. Typically, fully deprotonated carboxylates have equal bond distances between the Cδ and Oε1/Oε2 groups at ~1.26 Å. Within the CTX-M-14 SM23 complex structure, values somewhat between the above designated ranges for either a protonated or deprotonated carboxylate on residue 166 with the bond between the Glu166Cδ and Glu166Oε1 exhibiting a distance of 1.27 Å, and the distance between Glu166Cδ and Glu166Oε2 is 1.25 Å. Glu166 is protonated in this structure as can be seen with the F₀-F ≡ density (Figure 16); however, the bond distances in the carboxylic acid oxygen atoms and connecting carbon are showing closer distances characteristic of a resonating, deprotonated glutamate. This occurrence might theoretically be explained if this were an instance of a LBHB where the hydrogen is more delocalized and shared equally between the boronyl oxygen and the Glu166Oε1 oxygen, thus imparting more of a partial charge on Glu166Oε1. If the bond were partially delocalized, which is corroborated by the F₀-F ≡ density map, then having carbon-oxygen bond distances not conform to literature distances of 1.21/1.30 Å might be indicative of a fully protonated carboxylic acid. This partial delocalization of the proton between the ligand and catalytic residue could potentially be acting in a stabilizing fashion for the transition state. Before too many extrapolations are made, further evidence is needed to delve into the interaction between Glu166 and the boronyl moiety, with such techniques as QM/MM or NMR to verify the LBHB interaction or help explain the short bond distance with partially delocalized protons in the active site.

This 0.76 Å structure represents one of the highest diffracting β-lactamase crystal structures when compared to deposited structures in the Protein Data Bank. The structure of CTX-M-9 and SM23 has already been resolved to a resolution of 1.12 Å. The structure reported here
with CTX-M-14 shows a marked improvement where not only is the resolution greatly increased, but the ligand occupancy is also increased. The deposited structure 1YM1 did not resolve a single conformation of Lys73 to but rather had a mixture of states. The ligand occupancy was shown to be ~50% as the phosphate ion that is present in apo crystal structures had not been fully displaced and shared density with the carboxyl functional group on SM23 (Figure 18). In the data presented here, the occupancy is increased to nearly 100% and, as such, resolved a positively charged Lys73 and neutral Glu166, and while boronic acid transition state analogues are not exact replications of transition states, this structure helps shed light on the second half of the mechanism for class A β-lactamase hydrolysis.

Protein Engineering and Selective Active Site Permutations to Study LBHB Interactions

The first three projects within this chapter were concerned with looking at different states along the reaction coordinate to determine protonation of catalytic residues through the use of different types of ligands that isolate the enzyme at a specific stage of the mechanism. With this in mind, experiments were designed to determine if the active site of CTX-M-14 could be selectively perturbed through protein engineering in order to probe the potential LBHB interactions identified from the previously discussed crystal structures. The procedural route chosen to engineer in different, sequentially modified ligands incorporated the use of disulfide bonds. Protein disulfide bonds are covalent bonds between the sulfur atoms in the thiol groups of cysteine residues, where the bond forms when an oxidation reaction of the two thiol moieties links the residues together. The use of cysteine mutational analysis to form disulfide bonds has been well studied in the literature for a number of decades. Most of these studies used disulfide bond formation as a way to stabilize the enzyme for uses in biomedical or industrial applications that
could benefit from more stable, less labile enzymes.\textsuperscript{126} Incorporating disulfide bonds did not always increase the overall stability however, and would occasionally cause a decrease in stability.\textsuperscript{126}

From different mutational work that was performed with CTX-M-14, it was known that a tetrazole-based scaffold, such as J1X seen in Figure 19, will bind to CTX-M-14 readily, even if at a lower affinity, with structurally modest mutations to the active site. The hypothesis was, should a cysteine residue be incorporated in lieu of the catalytic residue Glu166, would it be possible to perturb the microenvironment of the active site selectively via different thiol ligands when forming the pre-covalent complex LBHB interaction?

The single mutant CTX-M-14 E166C was systematically generated, and then to allow for the thiol ligand to sit within the active site without steric clashes against neighboring residues, the double mutant CTX-M-14 E166C N170A was generated. The resulting CTX-M-14 double mutant readily crystallizes in either the P2\textsubscript{1} or P3\textsubscript{2}2\textsubscript{1} space groups and reaches full size in two weeks of growth. The preliminary crystal structure data shows that the double mutants readily diffract up to 0.85 Å resolution in the P2\textsubscript{1} space group and roughly 1.5 Å in P3\textsubscript{2}2\textsubscript{1}. For clarity, the preliminary structures presented here are in the P3\textsubscript{2}2\textsubscript{1} space group. While diffracting to a lower resolution that the P2\textsubscript{1} space group, P3\textsubscript{2}2\textsubscript{1} accommodates a slightly more accessible active site and allows for a larger ligand exchange to form disulfide bonds through soaking thiol ligands to react with the mutated residue Glu166Cys. The structures of the active site of WT CTX-M-14 apo enzyme, to CTX-M-14 double mutant with a fully formed disulfide bond while bound to a noncovalent tetrazole scaffold are presented in figure 20.

The structure for the double mutant enzyme with the thiol ligand sodium (1H-1,2,3-triazol-5-yl) sulfanide, shows and expansion of the active site when compared to the apo enzyme seen in Figures 20 B and A. As stated, the E166C N170A mutant was grown in the P3\textsubscript{2}2\textsubscript{1} space group at
resolution 1.50 Å and had $R_{\text{work}}/R_{\text{free}}$ values of 22.37%/24.81% respectively. This structure proves that through a soaking protocol I was able to generate the disulfide bond while still retaining the structural integrity of the active site. Also, the thiol soaking protocol did not have any off-target effects insomuch that residue Cys69, which is directly behind Ser70 in the active site, was not impacted by the thiol ligand. This is most likely due to Cys69 being buried underneath the β3 strand and not readily exposed to bulk solvent. The double mutant and binding of the thiol into the active site has a marked impact on the distances between the catalytic residues Ser70 and Lys73 when compared to the WT apo enzyme with PDB 4UA6 as a reference. In the WT enzyme, the bond distance between Lys73Nζ and Ser70Oγ is 2.75 Å, while in the mutant-thiol structure, the distance is 4.09 Å. The other residues surrounding the two unmutated catalytic residues Ser70 and Lys73 have minimal fluctuations, and the movement of Ser70 away from Lys73 by 1.34 Å might be explained by needing to accommodate of the somewhat bulky thiol ligand.

After obtaining a crystal structure of the CTX-M-14 double mutant E166C N170A with the disulfide bond formed on residue 166, the next experimental step was to obtain a complex structure with a tetrazole scaffold known to induce the pre-covalent active site conformation between residues Ser70 and Lys73. The resulting structure was crystallized in the P3$_2$2$_1$ space group at resolution 1.47 Å with $R_{\text{work}}/R_{\text{free}}$ values at 24.44%/27.54% respectively. The structural impact of J1X (Figure 19) binding, mostly concerns the distance between Ser70 and Lys73 as well as movement of the triazole ring. The triazole ring is compressed into the active site cavity and is within hydrogen bonding distance between Ser70Oγ and the N1 position on the triazole ring at 2.70 Å compared to the 3.33 Å in the apo structure. Upon ligand binding, the distance between Lys73Nζ and Ser70Oγ is decreased to 2.57 Å from the 4.09 Å. When comparing to a CTX-M-14 WT complex structure with the same tetrazole scaffold (PDB 6OOJ), the bond distance is 2.53 Å (Figure 21). The difference of 0.04 Å between the WT enzyme and the disulfide double mutant
enzyme is potentially within error at the resolution of 1.50 Å. This recovery of the short hydrogen bond distance between Ser70 and Lys73 for a potential LBHB interaction could lead to exciting ramifications for selective modifications to the microenvironment of the active site. In future work the thiol ligand could be modulated in a series of increasing polarity or charge to investigate the impact on the pKa of both catalytic residues in the pre-covalent complex.

Conclusions

This work presents the some of the first neutron crystal structures for WT CTX-M-9 in the apo form as well as in the pre-covalent complex. Each structure showcased the protonation state of the key catalytic residues. The perdeuterated pre-covalent crystal structure represents one of the highest diffracting neutron crystal structures currently on the PDB and highlights potential ramifications of room temperature formation of LBHB interactions. The next structure presented is the sub angstrom x-ray crystal structure of the deacylation transition state complex with CTX-M-14 and SM23. At 0.76 Å this structure is one of the highest resolution β-lactamase crystal structures that is on the PDB and resolves nearly all hydrogen atoms in the entire system. Notably, there can be seen a potential LBHB between the residue Glu166 and the boronyl oxygen atom. Lastly, the impact of sequentially altering the microenvironment on LBHB formation in the pre-covalent complex of CTX-M-14 by using thiol ligands to engineer disulfide bonds with cysteine mutants is investigated.
Experimental Procedures

Construct Design of CTX-M-9 and CTX-M-14

The codon optimized CTX-M-9 gene was synthesized and cloned into the plasmid vector PJ401. The resulting vector was transformed into competent BL21(DE3) *Escherichia coli* and plated onto lysogeny broth agar plates supplemented with 50 μg/mL of kanamycin for selection. For the CTX-M-14 protein, the encoding genes were cloned into a modified pET-9a expression vector, which was then transformed into XL-Gold cells for DNA sequencing. After DNA the sequence was verified, the vector was isolated using a Qiagen Midi Kit and transformed into BL21 (DE3) competent cells for expression and purification.

CTX-M-9 and CTX-M-14 Expression and Purification

From glycerol cell stocks stored at -80 °C, a Luria broth (LB) plate supplemented with 50 μg/ml kanamycin was streaked and incubated at 37 °C for 15 hours. A single colony was used to inoculate 50 ml of LB media containing 50 μg/mL of kanamycin for initial overnight culture. One liter of 2XYT media (16 g tryptone, 10 g yeast extract, and 5 g NaCl) supplemented with 0.2M sorbitol, 5mM betaine, and 50 μg/ml kanamycin was inoculated with 10 ml of cells for a 1:000 dilution. The cells were then incubated at 37 °C with shaking at 225 RPM. The optical density was monitored at 600 nM until a density of 0.3 was reached, and protein overexpression was induced with 0.5mM IPTG for 24 hours at 20 °C in a shaker at 250 RPM.

Cells were then harvested via centrifugation at 12,000g for 15 minutes at 4 °C and resuspended in lysis buffer of 20mM MES pH 6.0 supplemented with Pierce Protease Inhibitor EDTA free cocktail tablets. Cellular disruption was performed with sonication using 10 seconds ON and 15 seconds OFF at amplitude 10, for a total of 15 min and clarified with centrifugation at
50,000 RPM for 1.5 hours at 4 °C. The supernatant was dialyzed against 20mM MES pH 6.0 overnight at 4 °C. Initially using ion-exchange chromatography, the enzyme was purified using an Amersham Pharmacia Biotech CM Fast-Flow column and eluted with a linear gradient of 0.0 to 0.15M NaCl. The fractions containing the enzyme were then concentrated using a 10kDa Millipore Amicon filter. The enzyme was then loaded onto a GE Healthcare HiPrep 16/60 Sephacryl S-300 HR column for size exclusion with buffer 10mM Tris pH 7.0, 50mM NaCl, and 2mM EDTA. The resulting peaks were collected and concentrated to 20mg/ml and assayed to be greater than 95% pure using Coomassie Blue staining after sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Production of Perdeuterated CTX-M-9 Enzyme

The process of generating perdeuterated enzyme is an involved process that initially requires many nuanced steps to produce fully labeled proteins. Prior to any expression of enzyme, the cells need to be acclimated to growth conditions in minimal media where all hydrogen sources are replaced with dueterium. The minimal media composition includes: (NH₄)₂SO₄ at 6.86 g/L, KH₂PO₄ at 1.56 g/L, Na₂HPO₄·2H₂O at 6.48 g/L, (NH₄)₂-H-citrate at 0.49 g/L, MgSO₄·7H₂O at 0.25 g/L, D₂-glycerol at 5.0 g/L and a trace metal solution consisting of 0.5 g/L CaCl₂·2H₂O, 16.7 g/L FeCl₃·6H₂O, 0.18 g/L ZnSO₄·7H₂O, 0.16 g/L CuSO₄·5H₂O, 0.15 g/L MnSO₄·4H₂O, 0.18 g/L CoCl₂·6H₂O, 20.1 g/L EDTA. All media, plates and antibiotic solutions are created with D₂O.

Cells from a -80 °C glycerol stock that were initially grown in LB broth were used to streak a plate for a single colony. One colony was used to inoculate media supplemented with 50 ug/ml kanamycin and 50 ug/ml of ampicillin. The cells are slowly acclimated to sequential conditions of increasing deuterated minimal media with cultures being allowed to mix in ratios of 25% deuterated media/75% hydrogenated LB media for the first culture, a 50%/50% ratio for the second
culture until the growth media is 100% deuterated minimal media. Cell growth in the minimal media is much slower than in hydrogenated media, and as such the cultures would occasionally require more than 24 hours to grow to a cellular density reading of 2.0 at 600 nm with shaking at 250 RPM and 37 °C. Cells required monitoring for 24 to 72 hours to reach cellular density necessary to inoculate the next higher ratio of deuterated media.

To increase the yield of produced enzyme, a bioreactor was used for cell growth instead of the standard flask approach. All equipment or glassware that came into contact with the deuterated media and cells were autoclaved and then dried overnight in an oven to minimize the contamination via condensation or any residual moisture in the air. Directly from the oven, a 1.25 L vessel was attached to the bioreactor as well as outlet and inlet cords needed to add in buffering components to maintain a near constant pH optimal for growth while the cells proliferated. The vessel was then filled with deuterated growth media through an inoculation port. A sterilized feed port was also used to add D\textsubscript{8}-glycerol when levels dropped below optimal levels for cell growth and protein production. An air flow sensor was used to make sure the aeration of the media stayed at 0.5 L/min in conjunction with an agitation rate of 200 RPM. The medium was then inoculated with the D\textsubscript{2}O adapted cells. As the cells start to proliferate and consume the carbon source of D\textsubscript{8}-glycerol, the pH is monitored by an attached computer to regulate the addition of deuterated acid/base to maintain the pH within 0.1 of the initial 7.0 of the minimal medium. As a note, the consumption of the D\textsubscript{8}-glycerol results in a lowering of the pH, which can be used to monitor the relative rate of D\textsubscript{8}-glycerol uptake and feeding rate. Periodically the cells are checked at 600 nm for overall cell density and prevent over growth. The rate of carbon source being added to the media is carefully controlled to have the cells maintain a near constant growth rate. Since there is a maintained growth rate, the cells were induced to overexpress CTX-M-9 after a day of growing in the reactor. The fed-batch is allowed to continue for approximately five days to yield ~1g of cell
paste per gram of D$_2$-glycerol carbon source. The cell paste was fractionated into four tubes for ease of purification and prevent over-loading of the columns. Since the enzyme is perdeuterated the only deuterium that would be lost from exposure to hydrogenated buffers are the labile protons, the enzyme was purified according to the standard protocol listed above. Any lost deuterium atoms were regained through D$_2$O exchange after crystallization. From 1.25 L of growth media, ~120 mg of purified enzyme was generated.

**Mutagenesis of CTX-M-14 E166C**

To make the CTX-M-14 mutants – E166C, N170A, and double mutant E166C/N170A - the QuikChange Lightning Site-Directed Mutagenesis Kit was used.

**Crystallization of CTX-M-9**

Initial crystals were obtained using the hanging drop vapor diffusion method and grown at 20 °C with vacuum grease crystallization plates and siliconized coverslips from Hampton Research. Using micro seeding of crystals containing the P2$_1$ space group, 2 μl of reservoir solution 1M Na/K phosphate buffer pH 8.3 was combined with 2 μl of 20 mg/ml of CTX-M-9 to set up the crystallization condition. In approximately 14 days, fully formed crystals were present.

To grow crystals adequate for neutron diffraction experiments on the millimeter scale, Hampton 9 well glass plate and sandwich box approach was used. Larger scale drops were utilized in the sitting drop vapor diffusion approach with 20 μl of reservoir solution 1M Na/K phosphate buffer pH 8.3 combined with 20 μl of 15 mg/ml of CTX-M-9 for 40 μl total volume. The well was allowed to incubate for 2 days at 20 °C before seeding at 20 °C. When seeding, a serial dilution of 1:10,000 obtained by using a Hampton Research whisker seeding tool was used with freshly ground crystals from the Hampton hanging drop trays to facilitate only 1 or 2 crystals per well.
being formed. The trays were allowed to incubate at 20 °C for roughly two months for fully formed rectangular crystals with dimensions of ~ 1.5 x 0.5 x 0.5 mm.

**Sample Preparation of 3GK and SM23 complexes with CTX-M**

For the complex of 3GK and CTX-M-9, the crystals were allowed to grow to full size within two to three months due to the large size. Once the samples were done growing, the crystals were carefully transported to a new sitting drop well in the Hampton sandwich box set up with 5 μl of well solution (1 M Na/K phosphate pH 8.3) and 5 μl of 10 mM 3GK ligand for a total drop volume of 10 μl. The crystals were allowed to soak with the ligand for 24-48 hours with monitoring to harvest before cracks or degradation were noticed.

For the complex of SM23 with CTX-M-14, the crystals used were on the order of ~500 microns. The crystals were moved from the growth drop into a drop containing 1.44M sodium citrate to help facilitate the removal of the phosphate from the active site. The citrate also contained 10 μM SM23, which, once the phosphate was removed from the active site, was able to fully occupy the active site. Please note, CTX-M-14 crystals do not withstand long periods of soaking in 1.44 M citrate and were only soaked with 10 μM SM23 for six hours prior to harvesting.

**Sample Preparation for CTX-M-14 E166C/N170A Disulfide Bond Complex**

Initial crystals were grown using the seeding method as previously stated with buffering conditions using 1M K/Na phosphate buffer at pH 8.3 for the well reservoir solution. The purified point mutant of CTX-M-14 was used at 20 mg/ml and set up using the hanging drop method with siliconized cover slips. Using seeds with the space group P3\(_{2}2_1\), the crystals fully formed in one week. Using fully formed crystals, with care to avoid twinned crystals as the P3\(_{2}2_1\) space group is prone to twinning, the crystals were put into a new adjacent drop on the cover slip that contained
10 mM thiol ligand (sodium (1H-1,2,3-triazol-5-yl)sulfanide). The crystals were soaked with the thiol ligand for three to five hours and then transferred into a new drop containing 10 mM of the tetrazole ligand J1X. The crystals were allowed to soak with the tetrazole ligand for 24 hours and then placed into a drop containing 30% sucrose as a cryo protectant. The crystals were then flash frozen and sent off for data collection.

**Sample Preparation for Neutron Studies: D$_2$O Exchange Deuteration**

To increase the neutron scattering of the CTX-M-9 crystals, H/D exchange was performed through vapor diffusion within quartz capillary tubes. Deuterated crystallization buffer was prepared using D$_2$O to make 1M Na/K phosphate. Crystals were transferred into VitoTube 2.00 quartz capillary tubes and had the non-deuterated solvent removed using Hampton Research wicks to minimize the background scattering and more fully exchange labile H atoms for D. 20 µl of deuterated crystallization buffer was added to the end of the capillary tube and sealed using Hampton Research Capillary Wax. The H$_2$O/D$_2$O vapor diffusion exchange of labile protons occurred for a week before the start of data collection.

**Room Temperature Neutron Data Collection**

Neutron diffraction data were collected using the Macromolecular Neutron Diffractometer (MaNDi) instrument at the Spallation Neutron Source (SNS) using the time of flight (TOF) technique to give wavelength-resolved Laue diffraction data. The crystal was held stationary at room temperature, and diffraction data were collected for 20 hours using neutrons between 2.00-4.16 Å. Then the crystal was rotated by $\Delta \phi = 10^\circ$ and a subsequent data frame was collected. A total of 26 data frames were collected to form the neutron dataset. Diffraction data were reduced using the Mantid package, with integration being done by
three-dimensional TOF profile fitting. \textsuperscript{134, 135} Wavelength normalization of the Laue data was performed using the Lauenorm program from the Lauegen suite. \textsuperscript{136} The neutron data collection and processing statistics are given in Table 2.

**X-ray Data Collection and Structure Determination**

X-ray diffraction data for CTX-M-14 complexes and apo structures were collected using 19-ID and 22-ID-D beamlines at the Advanced Photon Source (APS) at Argonne National Lab. The data sets were indexed and integrated using the software iMosflm and then scaled using the program Scala housed within the CCP4 program suite. \textsuperscript{137, 138} All molecular replacement was performed using the software Phaser in the Phenix program suite with the initial model of the apo CTX-M-14 crystal structure (PDB 4UA6). \textsuperscript{139} The program phenix.refine was used for structure refinement, while model building was accomplished using WinCoot. \textsuperscript{140, 141} When fitting ligands for the neutron tetrazole complex and boronic acid inhibitor complex, the program Elbow was used to generate geometry restraints with classical parameters under a protonation state at pH 7 to be used with modeling and refinement. \textsuperscript{142} All associated figures were generated using the program PyMOL.
**Figure 5. Mechanism of Class A β-lactamase.** Electron Transfer is indicated with arrows. A) Substrate binding. Activated Ser70 attacks the β-lactam ring carbon. B) Tetrahedral acylation transition state (TS): Ser70 is stabilized by a short HB with Lys73, which is indicated with dashed lines. C) Pre-acyl-enzyme covalent intermediate: Proton transferred from Glu166 to Lys73. D) Acyl-enzyme complex: Glu166 activates the water to attack the acyl-enzyme bond. E) Deacylation TS: Lys73 protonates Ser70, followed by a proton transfer from Glu166 to Lys73. F) Product complex.
Figure 6. X-ray and Neutron Density Comparison of CTX-M-9. Here I have presented example spectra of residue Trp251 in CTX-M-9 of a combined x-ray/neutron data collection. Both x-ray and neutron data were collected to and refined at 1.7 Å. A) X-ray density with a sigma cutoff at 1.5 for the 2Fo-Fc map. B) Neutron density presented with a cutoff of 1.0 sigma with the 2Fo-Fc map. C) Combined x-ray/neutron density highlighting atomistic detail.
Figure 7. Crystal of CTX-M-9 Used for Neutron Diffraction with Maximum Dimensions of ~0.5 x 0.5 x 1.5 mm
Figure 8. Active Site Residues of CTX-M-9 with 2Fₒ-Fₑ Density Displayed with 1.5 σ. Protonation states of all key residues involved in hydrolase activity clearly shown in the Apo state.
Figure 9. Superimposed Structures of CTX-M-9 Neutron structure in Green and Ultra-High-Resolution Structure of CTX-M-14 in blue (PDB ID 4UA6). Relevant active site residues have been labeled.
### Table 2. CTX-M-9 Apo Neutron Data Collection Statistics

**Data Collection**

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**Refinement**

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* Values in parentheses represent highest resolution shells
Figure 10. Ligand Density within Perdeuterated Neutron CTX-M-9 3GK Complex. The ligand is shown at 90% occupancy within the active site of CTX-M-9. The x-ray density is presented in blue, while the neutron density is presented in red. A) X-ray diffraction data with $2F_o - F_c$ map contoured at 1.0 $\sigma$. B) Neutron diffraction data with $2F_o - F_c$ map contoured at 1.5 $\sigma$. C) X-ray and Neutron diffraction data combined.
Figure 11. Superposition of X-ray and Neutron Complex with Compound 3GK. The sub
angstrom x-ray data set used for comparison was taken from PDB 4UAA. The neutron structure
was collected using room temperature ~297 K, while the x-ray structure presented here was
collected under cryogenic condition at ~77 K.
Figure 12. Hydrogen Bonding Network with Catalytic Residues and Waters within CTX-M-9 Complex with 3GK Tetrazole. Catalytic triad residues Ser70, Lys73 and Glu166 all sit directly below the noncovalent inhibitor 3GK, which was left out for clarity.
Figure 13. Electron and Neutron Density of CTX-M-9 Active Site Residues in Tetrazole Inhibitor 3GK Complex. The x-ray and neutron density are presented separately and then together to help differentiate. X-ray density is presented in blue, while neutron density is presented in magenta. As the ligand 3GK noncovalently binds to the active site directly above the presented residues, the ligand was left out of the figure. A) X-ray $2F_o-F_c$ density contoured at 1.0 $\sigma$. B) Neutron $2F_o-F_c$ density contoured at 1.5 $\sigma$. C) Combined x-ray and neutron $2F_o-F_c$ density.
Figure 14. Boronic Acid Transition State Analogues. SM23 complexes with CTX-M-14 to form the deacylation transition state while CB4 complexes with CTX-M-14 to trap the enzyme in the acylation transition state conformation.
Figure 15. Electron Density of SM23 with CTX-M-14. The 2F_o-F_c electron density is contoured at 1.0 σ with a resolution of 0.76 Å.
Figure 16. Active Site Density of CTX-M-14 SM23 Complex. The $2F_o-F_c$ density shown here in blue is contoured to 1.5 $\sigma$, while the unbiased $F_o-F_c$ density shown in red is contoured at 2.5 $\sigma$. For clarity, the ligand was truncated to only show the boron connectivity.
Figure 17. Focused Interaction between Glu166 and Ligand Boronyl in CTX-M-14 SM23 Complex. The bond distance between Glu166 and the oxygen atom of the boronyl group is 2.47 Å. The $2F_o-F_c$ map is contoured at 1.5 $\sigma$, while the $F_o-F_c$ map is contoured at 2.5 $\sigma$. 
Figure 18. Superposition of CTX-M-14 and CTX-M-9 SM23 Complexes. The protein in blue is the work presented here in this chapter, while the magenta enzyme is from the PDB 1YM1.
sodium (1H-1,2,3-triazol-5-yl)sulfanide  J1X

Figure 19. Thiol Disulfide Bond Former and Tetrazole Scaffold for Complex Formation.
Figure 20. CTX-M-14 Progression from WT Apo to Double Mutant Disulfide Bond Complex. A) CTX-M-14 WT enzyme crystal structure. B) CTX-M-14 E166C N170A double mutant crystal structure with disulfide bond formed with sodium (1H-1,2,3-triazol-5-yl)sulfanide.
Figure 20. Continued. C) CTX-M-14 E166C N170A disulfide bond complex with tetrazole based scaffold J1X in active site.
Figure 21. Superposition of CTX-M-14 E166C N170A Disulfide J1X Complex with CTX-M-14 WT J1X Complex. The structure shown in green is the data presented here, while the magenta structure is from the PDB 6OOJ.
Chapter 3:

Structural Verification of Tetrazole-Based Scaffolds Used to Investigate Amide - Heteroarene Pi-Stacking in the Inhibition of Class A β-lactamase

Overview

In collaboration with the Renslo Group of UCSF, structural credence was needed in order to corroborate the kinetic data surrounding the systematic study of modified heteroarene tetrazole scaffolds interacting with the amide backbone of the β3 of CTX-M-14 and CTX-M-27. This study delved into the little studied area of heteroarene–amide π stacking to be utilized as a tool for inhibitor design in structure-based drug discovery. There have been several computational studies that look to calculate the heteroarene-amide interaction but lack experimental protein systems to validate the calculations. In this effort, I resolved five crystal structures of the class A β-lactamases CTX-M-14 and CTX-M-27, and targeted the interactions of the Gly238 amide on the β3 strand with tetrazole-based scaffolds to monitor placement of the heteroarene probe. The resulting data resolved remarkably consistent placement of the scaffolds, thus allowing for extrapolations to be made concerning the inhibitory data and change in free energy associated with the sole difference being dictated by the heteroaryl substituent.

Introduction

Computational chemists and biologists have been interested in trying to use heteroarene-amide interactions as a potential tool to use in drug design as well as to be incorporated into current scoring schemes/force fields associated with intermolecular interactions. This interaction was first
noticed when looking at the stacking of aromatic side chains on the amides of the protein backbone where a computational group mined large crystallographic data sets. The implication that the heteroarene-amide interactions between ligands and protein binding were an important area and untapped resource.

My contribution to the collaboration relied initially on previously resolved crystal structures that were utilizing fragment-based drug design to identify tetrazole-based scaffolds (Figure 22) as inhibitors against the class A β-lactamase CTX-M-9. Combined with subsequent iterations of the tetrazole scaffold that were designed to increase activity, it can be seen that there is a concerted binding mode when all scaffolds are superimposed (Figure 23). The extensive hydrogen bonding network is maintained between the ligand and protein for the example tetrazoles shown in Figure 23 and lends validity that the scaffold is a potential starting point for systematic development of different functional groups to probing the amide-stacking on Gly238. In addition to CTX-M-14, the enzyme CTX-M-27 was also employed, where the enzymes only differ in the active site by residue 240 (Figure 24). While the two enzymes do have distinct antibiotic profiles, the premise for the use of CTX-M-27 in conjunction with CTX-M-14 in this case, is residue difference at 240 where in CTX-M-14 residue 240 is an aspartate and in CTX-M-27 the residue is a glycine (Figure 24). The use of CTX-M-27 reduces potential steric clashes when investigating ligand pi stacking on the backbone of residue 238 and makes analysis of potential energies associated with ligand binding easier to analyze.

Herein it is shown that the hydrolase enzyme CTX-M is a good predictive model for the study of amide-heteroarene pi stacking under physiologically relevant conditions. The highly conserved interactions and binding mode of the tetrazole scaffold includes polar and hydrophobic interactions, extensive hydrogen bonding, and stacking of the tetrazole group with the β3 strand and hydrophobic interactions between residue Pro167 and the trifluoromethyl group. These
findings can be applied to theoretical calculations and predictions for future development of more accurate, inclusive energy assessments for protein-ligand interactions to be used in inhibitor design.

**Results and Discussion**

In order to assess as to whether these tetrazole scaffolds presented in Figure 25 bind to CTX-M in the predicted manner and stack accordingly to Gly238 on the β3 strand, I solved representative complexes with both CTX-M-14 and CTX-M-27. Of the five crystal structures that were collected and report here, three are complexes with CTX-M-14 and two are of complexes with CTX-M-27. Looking to Figure 25 as a scheme for the synthetic compounds the Renslo group prepared, representative complexes included compounds 3, 14 and 20. Just to reiterate, the CTX-M isoforms differ in the active site only by one position at 240 on the β3 strand, directly adjacent to Gly238 as by naming convention for this system in class A β-lactamases there is no residue 239. This residue directly next to the amide-heteroarene site is an aspartate in CTX-M-14 and a glycine in CTX-M-27. Within CTX-M-14, x-ray diffraction complexes with ligands 3, 14 and 20 were solved to resolutions of 1.4 Å, 1.4 Å, and 1.25 Å, respectively. These crystal structures show density corresponding to a highly conserved binding orientation, where the heteroarene substituent all interact in an apparent stacking interaction with Gly238 (Figure 26). Of note, the ligands were modeled into the unbiased density Fo-Fc map at a contour of 3σ, showing every atom in the scaffold with nearly 100% completeness for each ligand. When looking at residue Asp240, we can see that in all three CTX-M-14 complexes, there is potential steric clashing occurring, where the R group of Asp240 is pushed away from the active site and into the bulk solvent to accommodate the ligand and stacking interactions. The compounds 14 and 20 were also solved in complex with CTX-M-27 with respective resolutions of 1.5 Å and 1.25 Å (Figure 27). The tetrazole ligands adopted a
similar binding pose as predicted based on the obtained structures with CTX-M-14. The unbiased
$F_o-F_c$ density map contoured at 3$\sigma$ show full ligand occupancy for these two structures.

Looking to the statistics in Table 3 governing the five reported complexes, we can see that
the overall completeness of each structure ranges from 87.8% to 99.9%, thus lending to confidence
to the model interpretation. B factors can be interpreted as atomic displacement factors or thermal
fluctuation factors and can be used to help dictate or have greater certainty about atomic position.

The lower the B factor, the less thermal fluctuation, or more precisely stated, the higher the
confidence about the modeled location of the atoms in the x-ray structure. The B factors listed
for each protein ranges from 12.10 to 15.84, highlighting a stable protein system. Values of 60 or
greater can typically be inferred to be disorder in the particular residue in question and may indicate
multiple conformations or high motility. When looking at the B factors for the ligands, we see
a similar range of numbers between 14.73 and 18.57 for compounds 14 and 20 in both CTX-M-14
and CTX-M-27. The exception is compound 3 in CTX-M-14, which has a B factor of 29.83, and
may be explained by steric involving Asp240 as compound 3 is a six membered ring compared
to the five membered rings seen in compounds 14 and 20. Even with the higher-than-average B
factor, compound 3 still adopted the predicted conformation in the active site and exhibited the
intended stacking interaction with Gly238 (Figure 26). The amide-heteroarene stacking distances
can also be seen within all five complexes and have a range of 3.7 to 3.9 Å (Figure 28). The
distance was measured from the center of the density of the heteroarene ring to the midpoint on
the amide nitrogen atom. These values are all within accepted ranges for the associated
heteroarene-amide pi-stacking interactions.

Lastly, needs to be mentioned that with tetrazole scaffolds that contain asymmetric
heteroarene rings, there are two rotomeric states available to be adopted when stacked on residue
Gly238. In order to have complete certainty about the directionality of the ring, higher resolution
structures are needed to interpret the correct rotomeric state via the differences in the bond
distances within the ring, which can be assigned using sub angstrom crystallography.

Conclusion

Herein I have shown crystallographic data validating a new model for predicting amide-
heteroarene pi stacking interactions. The advantages of using this model include the binding of the
ligand being reversible in a non-covalent fashion that could allow for a wide variety of different
heteroarene ligands to be substituted. Even when the ligands show higher than average B factors,
the model is highly predictable with a conserved binding mode allowing for even slightly
unfavorable ligands to be used. The CTX-M protein system readily crystallizes in different space
groups and conditions, thus allowing for a wide range of conditions should potential ligands need
a unique environment to obtain a complex crystal structure. Lastly, CTX-M can be used to obtain
sub angstrom resolution data sets that can elucidate the different states of unsymmetrical ligands.

Experimental Procedures

Purification of CTX-M-27

CTX-M-27 has a similar purification protocol to the one listed in Chapter 2. Briefly, the
enzymes were expressed using the BL21 (DE3) cell line with the protein encoded in the Pet9A
vector. Cells were grown on LB agar plates supplemented with 50 μg/ml kanamycin from cell
stocks stored at −80 °C. Single colonies were used to inoculate 50 ml of LB broth with 50 mg/ml
kanamycin and grown at 37 °C over- night. From the overnight culture, 10 ml of cells were used
to inoculate 1 L of 2XYT broth CTX-M-27. The cells were grown at 37 °C until an OD600 of
0.5 to 0.8 was reached. Overexpression of protein was induced with the addition of 0.5 mM
isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20 °C for 24 hours, and the cells were harvested with centrifugation at 5000 RPM for 15 minutes at 4 °C.

CTX-M-27 cell pellets were resuspended in 50 mM MES pH 8.0 with 2 mM EDTA. Cells were lysed with sonication and cellular components separated via ultracentrifugation at 45000 RPM for 1 hour. The protein was loaded onto a CM Sepharose column and eluted with an increasing NaCl gradient. Enzymes were additionally purified using a size exclusion HiLoad 16/60 Superdex 75 column. Final protein purity was evaluated with SDS-PAGE to be at or greater than 95%.

**Crystallization of CTX-M-27**

Similar to CTX-M-14, the protein CTX-M-27 readily crystallizes in the P2₁ space group. These crystals were grown using the hanging drop method with roughly 20 mg/ml enzyme concentration mixed in 1:1 ratio with well solution of 1M potassium phosphate pH 7.9. The drops were seeded to facilitate quicker growth and allowed to grow for 1 to 2 weeks to allow for full crystal size to be achieved.

**Heteroarene Ligand Complex Preparation**

For both CTX-M-14 and CTX-M-27 complex structures were generated by soaking 5 – 10 mM ligand concentrations with protein crystals for 3 - 24 hours in 1 M potassium phosphate pH 7.9 or 1.44 M sodium citrate prior to cryoprotecting with 30% (wt/vol) sucrose supplemented crystal mother liquor.
X-ray Data Collection and Structure Determination

Crystal diffraction data sets were collected at the beamlines 22-ID, 22-BM, and 19-BM at Argonne National Laboratory Advanced Photon Source (APS). The data sets were indexed, integrated, and scaled using the program HKL2000. Initial models were obtained via molecular replacement with the program Phaser in the Phenix suite. Refinement was carried out using phenix.refine, and ligand restraint files were generated with the program Elbow. The $F_o-F_c$ and $2F_o-F_c$ maps were generated with the program phenix.mtz2map program for all structures.

Note to Reader #2


Note to Reader #3

All compound synthesis work and kinetic analyses were performed by the Renslo Lab at UCSF.
Figure 22. Precursor Tetrazole Scaffolds Found Through Fragment Based Drug Discovery. The compound F13 is from PDB 3G35 and compounds DN6 and DN8 are from PDBs 4DE3 and 4DDY respectively.
Figure 23. Superposition of Precursor Fragment Based Drug Design Derived Tetrazole Scaffolds. Overlay of ligands F13, DN6 and DN8 all showing a maintained hydrogen bonding network and minimal movement regardless of ring substitution.
Figure 24. Superposition of CTX-M-14 and CTX-M-27 β3 Strand. The structure in blue corresponds to CTX-M-14, and the structure in green is CTX-M-27.
Figure 25. Structure of CTX-M Inhibitors. Red arrows correspond to dipole moment of heteroaryl ring.
Figure 26. Complex Structures of CTX-M-14 with Compounds 3, 14 and 20. Top figure is compound 3 from PDB 6OOK. Middle figure is compound 14 from PDB 6OOJ. Bottom figure is compound 20 from PDB 6OOF. Ligands are modeled into the unbiased $F_o$-$F_c$ density contoured at $3\sigma$. Stacking distances are listed above the heteroarene measured from the centroid of the ring to the nitrogen atom in the amide.
Figure 27. Complex Structures of CTX-M-27 with Compounds 14 and 20. Top figure is compound 14 from PDB 6OOH. Bottom figure is compound 20 from PDB 6OOE. Ligands are modeled into the unbiased Fₐ-Fₐ density contoured at 3σ. Stacking distances are listed above the heteroarene measured from the centroid of the ring to the nitrogen atom in the amide.
Table 3. Amide-Heteroarene Stacking X-Ray Data Collection and Refinement Statistics

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| Refinement | | | | | |
| Resolution (Å) | 35.3 - 1.236 | 30 - 1.26 | 25.71 - 1.4 | 29.92 - 1.499 | 25.27 - 1.4 |
| No. Heavy Atoms | | | | | |
| Protein | 4988 | 4206 | 4121 | 4131 | 4136 |
| Ligand/Ion | 116 | 116 | 145 | 145 | 125 |
| Water | 691 | 729 | 738 | 787 | 697 |
| B-Factors (Å²) | | | | | |
| Protein | 14.00 | 12.10 | 15.84 | 15.14 | 15.72 |
| Ligand/Ion | 16.12 | 14.73 | 18.57 | 18.37 | 29.83 |
| Water | 26.43 | 25.92 | 29.59 | 27.74 | 29.02 |
| Ramachandran Plot | | | | | |
| Most Favored Region (%) | 97.3 | 97.7 | 97.7 | 97.9 | 97.9 |
| Additional Allowed (%) | 2.32 | 1.93 | 1.93 | 1.73 | 1.74 |
| Generously Allowed (%) | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 |

* Values in parentheses represent highest resolution shells
Figure 28. Top and Side View of Heteroarenes Stacking on Gly238. Top and middle figure are compound 14 stacked from top and side views respectively. The bottom figure is Superimposed top view of 3, 14, and 20 with CTX-M-14.
Chapter 4:

Investigation of Resistance in CTX-M-14 Through Analysis of Bicarboxylate Pairs

Overview

The previous two chapters were concerned with function and inhibition of class A β-lactamase enzymes. The last portion of work presented will cover a potential resistance mechanism employed by CTX-M-14 through the employ of a strong, bicarboxylate pair adjacent to the active site of the class A β-lactamase. This particular occurrence was discovered in a previous study of CTX-M-14 where a sub angstrom crystal resolved the particular instance of a short, 2.47 Å hydrogen bond between residues Asp233 and Asp234. 32 This particular hydrogen bond was investigated by way of mutational analysis, crystallographic studies and melting temperature experiments to attempt to understand the importance of the interaction located so close to the active site on the β3 strand the sits directly above the active site.

Bicarboxylate Pairs in Proteins

Pairs of hydrogen-bonding carboxylate groups, especially between aspartate residues, are commonly found in protein structures. 91, 149 While such pairs do not form favorable interactions when solvent-exposed and at physiological pH (due to electrostatic repulsion), side chain carboxylates with perturbed pKa values inside the protein microenvironment can share a proton and establish strong hydrogen bonding contacts, including some very short (~2.5 Å) hydrogen bonds (HBs). Hydrogen-mediated bicarboxylates have been studied for their role in enzyme
catalysis, including the reaction catalyzed by HIV protease. However, a possible structural function of such bicarboxylate pairs has been much less investigated.

Pairs of highly conserved aspartate residues have been observed across many class A β-lactamases; bacterial enzymes catalyzing the hydrolysis of β-lactam antibiotics, e.g., ampicillin (Figure 1).150-152 One such pair consists of Asp233 and Asp246, two residues located outside, but in close proximity to, the active site. In many of these enzymes, the Asp233-Asp246 length has been shown to be short, in the range of 2.5-2.6 Å.146,152-155 Additionally, Asp233 resides on the β3 strand that forms part of the active site, including the oxyanion hole crucial to the stabilization of the reaction transition states. These structural features, together with the sequence conservation,146,153-155 suggest that the Asp-Asp HB may play a special role in the structure and function of class A β-lactamases, even though it is not directly involved in substrate binding or the chemical reaction.

CTX-M class A β-lactamase is the most common clinically observed ESBL and is capable of hydrolyzing third-generation cephalosporins (e.g., cefotaxime, Figure 29) and other common β-lactam antibiotics such as penicillins.19,124 It provides a well-characterized model system for investigating class A β-lactamase structure and catalysis. In a recent study of CTX-M β-lactamase using sub angstrom resolution x-ray crystallography, we have observed a short HB (2.47 Å) between Asp233 and Asp246, including a hydrogen atom that is apparently shared by the two carboxylate groups.32 In order to study the importance of the Asp-Asp pair in class A β-lactamase structure and function, we constructed several CTX-M mutants, and used x-ray crystallography and biochemical studies to gain a better understanding of the structural and functional contribution of this conserved interaction outside the active site. Interestingly, my results indicate that the Asp-Asp HB is important for stabilizing both the 213-219 loop and β3 strand in class A β-lactamases, which in turn are critical features for maintaining the active site integrity required for substrate recognition and catalysis. These results provide valuable insights into the contribution of Asp-Asp
pairs to protein structure and function, as well as the role of protein flexibility in the evolution of drug resistance.

**Results**

**Functional Analysis of the D233N Mutation**

To investigate the Asp233-Asp246 interaction, particularly its contribution to enzyme stability and activity, several mutants (D233N, D246N, D246I) were constructed using site-directed mutagenesis, in order to disrupt the short HB between the Asp-Asp pair. Both Asp233 and Asp246 were mutated to asparagine but D246N failed to fold properly during protein expression and we were only able to purify the D233N mutant. Because Asp246 is replaced by isoleucine in TEM-1 and SHV-2 class A β-lactamase, next the D246I mutant was investigated, which behaved similarly to the D246N mutant with the inability to properly fold. Since Asp246 is buried close to the tightly packed protein core, it is possible that both the D246N and D246I mutations may have caused steric clashes with surrounding residues. Although the result of D246N initially appeared surprising, close examination of the CTX-M wild type (WT) structure suggests that the additional hydrogen atom on Asn246-Nδ2 may clash with the Asn246-Cα group if the asparagine side chain adopts the same conformation as Asp246.

The studies subsequently focused on the CTX-M-14 D233N mutant. In order to gain a clear understanding of the importance of the Asp-Asp interaction with respect to enzyme function, I performed biochemical kinetic studies of the mutant and compared them with the WT CTX-M-14 enzyme (Table 4). The activity of the enzyme was tested using nitrocefin, ampicillin, or cefotaxime as substrate (Figure 29). Whereas nitrocefin is a standard substrate for assaying β-lactamase activity and may represent the early-generation cephalosporins, cefotaxime is a third-generation
cephalosporin and a so-called ESBL antibiotic. Its bulky side chain prevents it from being hydrolyzed by narrow-spectrum β-lactamases such as TEM-1 (Figure 29). However, CTX-M and other ESBLs have evolved to accommodate these antibiotics as substrates and efficiently catalyze their hydrolysis. Interestingly, the D233N mutant displayed $K_m$ and $k_{cat}$ values similar to WT when tested using the nitrocefin substrate. On the other hand, when tested with ampicillin and cefotaxime as substrate, the D233N mutant exhibited lower activity compared to the WT, with respectively a 3-fold and 6-fold reduction in $k_{cat}/K_m$. It was considered whether these substrate-dependent differences in $k_{cat}/K_m$ could be attributed to the effects of the D233N mutation in accommodating different substrates. Interestingly, for ampicillin, the change in activity derived primarily from a ~2-fold decrease in $k_{cat}$, whereas for cefotaxime, a ~7-fold increase in $K_m$ was observed. For the hydrolysis reaction catalyzed by class A β-lactamases, the deacylation process is usually the rate-limiting step. In these cases, $k_{cat}$ and $k_{cat}/K_m$ reflect the efficiency of deacylation and acyl-enzyme formation respectively, as described in previous studies. It appears that the D233N mutation had some small effect on the deacylation step for ampicillin, but a rather significant effect on the acylation reaction for cefotaxime (i.e., both substrate binding and the formation of the acyl-enzyme linkage).

To further probe the D233N mutation’s influence on ligand binding, the inhibition of the WT and mutant by a known non-covalent, tetrazole-based inhibitor was studied, (compound 1, Figure 29). The compound showed approximately 10-fold weaker inhibition of the mutant than the WT protein, with the $K_i$ value increasing to 226.6 µM from 21.4 µM. These results indicate that the D233N mutation directly impacts non-covalent interactions between the protein and ligand in the active site.

Through melting temperature experiments, it was studied as to whether the activity decrease may have resulted from change in protein stability. Surprisingly, the D233N mutant
displayed slightly increased protein stability compared with the WT (55.1±0.6 °C vs 54.3±0.5 °C, Figure 30), suggesting that the Asp-Asp interaction may be needed to maintain a local structural feature rather than the global stability.

**X-ray Crystallographic Structure of the D233N Mutant**

To understand the structural basis for the decrease in activity, and slight increase in stability, of the D233N mutant, the protein was crystallized. Using potassium phosphate buffer and microseeding methods, previous CTX-M proteins were crystallized in both the P2₁ and P3₂2₁ space groups. However, I was only able to crystallize D233N in the P3₂2₁ space group. These observations hinted at structural differences between D233N and WT. The WT CTX-M-14 apo crystal structure and the D233N mutant, both in the P3₂2₁ space group, were solved to 1.8 Å and 2.0 Å resolutions, respectively (Figure 31A and 31B). The two structures are nearly identical, with an r.m.s.d value of 0.216 Å when superimposing all residue atoms. However, there are significant differences near the active site, particularly the movement of residues 214-218 (in the 213-219 loop) and surrounding residues (Figure 4). Residues 214-218 shift ~0.9 Å away from the β3 strand, which consists of residues 231-240. The biggest movement (1-1.6 Å) is observed in the side chain of Thr216. These conformational changes also lead to smaller shifts (~0.4 Å) in nearby residues, including Tyr129 and Ser130, and the loss of the HB between Thr216 and Thr235 on the β3 strand, accompanied by a small movement of Thr235 as well. The residue movements may explain why the mutant could not be crystallized in the P2₁ space group, because some of these residues are involved in crystal packing in the P2₁ crystal form, but not the P3₂2₁ space group. In addition, the movement of Thr235, and to a lesser extent Ser130, may be responsible for the loss of the phosphate molecule (from the crystallization buffer) in the protein active site, as both Thr235 and Ser130 are involved in coordinating the phosphate. In previously determined structures,
Thr235 and Ser130 have also been found to interact with the C3(4)-carboxylate group of the β-lactam substrate. \(^{162, 163}\)

An examination of the structure at the Asn233-Asp246 position provides clues to the conformational shift of residues 214-218. In the mutant structure, the short hydrogen bond between Asp233 and Asp246 has been abolished, with the Asn233Nδ2 now within a standard hydrogen bonding distance of 3.0 Å with Asp246Oδ1 (Figure 4A). A series of other structural changes have also taken place due to the alteration of Asp233Oδ2 to Asn233Nδ2. In the WT structure, Asp233 is located close to Gly217, with Asp233Oδ2 placed 3.6 and 4.6 Å away from the Gly217Cα and Gly217O respectively. In the mutant structure, the distances between the corresponding Asn233Nδ2 and Gly217Cα/Gly217O have changed to 3.9/3.4 Å respectively, largely due to a shift in the Gly217 position as described above. I hypothesize that this change of contacts between Asp/Asn233 and Gly217 may be the trigger for the movement of residues 214-218 and originates from potential favorable and unfavorable interactions between Asp233Oδ2/Asn233Nδ2 and Gly217Cα/Gly217O. Asp233Oδ2 and Gly217Cα may be involved in a special CH •••O weak HB that has been well documented, particularly involving Cα atoms (even though its biological significance has been debated). \(^{164, 165}\) The distance between Asp233Oδ2 and Gly217Cα, and the locations of the oxygen lone pair and Hα atom, seem to support such a HB. Although the strength of this special HB may not be particularly strong, it is at least favorable. The interaction also places Gly217O away from Asp233Oδ2 and reduces electrostatic repulsion between the two partially negative charged atoms. In contrast, replacing the oxygen lone pair of Asp233Oδ2 with a hydrogen atom on AsnNδ2 can cause unfavorable interactions with the Hα atom of Gly217. The movement of Gly217 not only positions Gly217Cα away from Asn233Nδ2, but it also brings Gly217O closer to Asn233Nδ2, with a distance of 3.4 Å, allowing for favorable interactions and possibly a weak HB. Taken together, these potential favorable and unfavorable interactions, albeit individually
weak, may have led to the shift in the Gly217 position and the rest of the 213-219 loop. In addition, the D233N substitution has also weakened the HB with Asn214, with the length increasing from 3.3 Å between Asp233Oδ1 and Asn214Nδ2, to 4.0 Å (Figure 32A). This is accompanied by a slight rotation in the Asn214 end group, enabling it to form a HB with the backbone carbonyl group of Asp233, with a distance of 3.1 Å.

Analysis of the temperature factors (B factors) of the structure, an indicator of structural heterogeneity and thermal motion, also revealed increased mobility of the 213-219 loop and the catalytic water. The average B factors of the WT and D233N mutant structure are 29.2 and 34.0 Å² respectively, with the mutant value 4.8 Å² (16%) higher and consistent with its slightly lower resolution (2.0 Å vs 1.8 Å for the WT). In comparison, the B factor values of residues 213-219 are 19.2 and 36.9 Å² for the WT and mutant respectively, with the mutant value 17.7 Å² (92%) higher than the WT. A similar trend is observed for active site residues and the catalytic water, albeit to a lesser extent. Particularly, residues 235-237 have an average B factor of 11.1 and 15.9 Å² in the WT and D233N mutant respectively, with an increase of 4.8 Å² (43%). The catalytic water molecule exhibits a B factor of 8.5 and 13.2 Å² for the WT and D233N mutant respectively, an increase of 4.7 Å² (55%). The average B factors for water molecules are 37.4 and 31.6 Å² for the WT and mutant respectively, although these values do not provide a good reference point due to the smaller number of water molecules in the mutant structure resulting in a lower average B factor value.

**X-ray Structure of the D233N Mutant Complexed with Tetrazole Ligand Compound 1**

The complex crystal structure of compound 1 with CTX-M-14 WT was determined previously. To understand how the D233N mutation may affect ligand binding, we solved the complex structure of the D233N mutant with compound 1 to 1.95 Å resolution. Compared with
the D233N mutant apo structure, inhibitor binding has led to considerable conformational changes in the active site, restoring the Thr216-Thr235 HB observed in the WT–compound \( \text{I} \) complex. As shown in Figure 5, the binding of the tetrazole inhibitor moves Thr235 towards the active site to more closely resemble its position in the WT structure, establishing a HB between Thr235 and the tetrazole ring. This in turn causes changes in Thr216 and Gly217, moving Thr216 also to the location in the WT structure and enabling a HB with Thr216. In comparison to Thr216 and Thr235, Gly217’s position is different from those in the WT and D233N apo structures, and the density also appears to be slightly less ordered compared with the apo structures, suggesting possible alternative conformations.

**Functional Analysis of the T216A mutation**

As the D233N mutation caused conformational changes in the 213-219 loop, particularly the loss of a HB between Thr216 and Thr235, we next investigated the contribution of Thr216 to enzyme activity. Specifically, the T216A single mutant and the D233N/T216A double mutant were constructed using site-directed mutagenesis. The biochemical assay results demonstrated a significant decrease in activity for both mutants when tested against nitrocefin, ampicillin, and cefotaxime. Particularly, when compared to the WT, the activity of the T216A mutant exhibited approximately 4- and 7-fold higher \( K_m \) values against nitrocefin and ampicillin respectively. In the case of cefotaxime, conclusive results were unable to be obtained due to the dramatic increase in \( K_m \) values resulting in undersaturation of the enzyme, even with high substrate concentrations up to 1 mM. Meanwhile, the T216A mutation led to a ~3-fold increase in the \( k_{cat} \) value for nitrocefin, but no significant change for ampicillin. Consequently, there was a small (30%) decrease in \( k_{cat}/K_m \) for nitrocefin, and a more significant 5-fold reduction for ampicillin.
For nitrocefin, the increase in $k_{cat}$ is interesting and indicates a more efficient deacylation process. Nitrocefin is a unique cephalosporin substrate in that its C3 side chain does not dissociate after formation of the acyl-enzyme complex. Based on previous structures, this cephalosporin side chain interacts with Thr216 during the formation of the Michaelis complex. The T216A mutation may therefore affect substrate binding. It is also possible that the C3 side chain of nitrocefin, missing for other substrates in the acyl-enzyme complex, may restrict the conformational changes of the substrate required for the progression of the reaction. The T216A mutation can relieve these constraints in the acyl-enzyme intermediate, increasing the deacylation rate. For ampicillin, the T216A mutation appears to have minimal effect on the deacylation process but have a significant impact on the acyl-enzyme formation, probably due to its influence on substrate binding.

Interestingly, the trend in the activity change between D233N/T216A and T216A is similar to that between D233N and WT, suggesting that the effects of the D233N and T216A are somewhat independent of one another and can be synergistic. As the T216A mutation also affects the HB between Thr216 and Thr235, these results indicate that the influence of D233N on enzyme activity is more than impacting the contributions of Thr216 and Thr235 to substrate binding.

**Discussion**

The mutagenesis, biochemical, structural, and microbiological experiments described herein suggest a potentially important role of the Asp-Asp pair with respect to the functionality and stability of CTX-M specifically, and possibly in other contexts as well. These results have implications for understanding this conserved short HB interaction across class A β-lactamases, which also highlights the critical features of the 213-219 loop in maintaining active site integrity and function.
Thr216 and loop 213-219 in Class A β-lactamase function

These studies have shed light on the essential role of Thr216 and the loop containing residues 213-219 in the structure and function of class A β-lactamases. Previous complex structures have shown that Thr216 forms non-polar interactions with the substrate. However, this contribution to ligand binding alone may not explain the dramatic loss of activity in the T216A mutant. Thr216 hydrogen bonds with Asn214 and Thr235, and, like Asn214, forms a water-mediated interaction with Lys234 (Figure 31A). Lys234 and Thr235 are two highly conserved residues on the β3 strand, which lines one side of the active site and plays a crucial role in substrate binding and catalysis. Thr235 interacts directly with the C3(4)-carboxylate group of the substrate. Lys234 serves as an electrostatic anchor for the negatively charged substrate and may also influence the pKₐ of the nearby Lys73, a crucial catalytic residue hypothesized to function as the general base in the acylation reaction. The interactions between Thr216 and these residues may stabilize the active site configuration necessary for substrate binding and catalysis. In TEM-1 and SHV-2, Thr216 is replaced by a valine and Asn214 is substituted for an aspartate residue. Compared with Asn214 in CTX-M, Asp214 in TEM-1 and SHV-2 forms a new water-mediated interaction with Thr235, potentially strengthening the contact with Lys234, which is also mediated by a water molecule. The new interactions again maintain a strong linkage between the 213-219 loop and the two important active site residues of Thr235 and Lys234.

In addition to the contacts with the β3 strand, Thr216 and residues on the 213-219 loop also interact with other structural elements contributing to the active site. The Cγ2 atom of Thr216 is in van der Waals contact with Tyr129Cα and Tyr129C, whereas the side chains of Thr215 and Tyr129 form non-polar interactions with one another (Figure 32B). Both the backbone and side chain of Thr215 form HBs with Gln128O. Furthermore, Gly213-Asn214 have extensive van der
Waals contacts with Gln128. These interactions may help position Ser130 for substrate binding and general acid catalysis during the acylation step, two roles suggested by previous studies 170-172.

The 213-219 loop connects two α-helices (residues 200-212 and 220-225) (Figure 32B). The two helices interact extensively with the β3 strand and the α-helix that contributes the catalytic Ser70 and Lys73. This expansive network of molecular interactions suggests that the 213-219 loop may play a crucial role in ensuring a productive configuration of the active site.

**The Asp233-Asp246 pair in Class A β-lactamases**

Asp233 is highly conserved in class A β-lactamases, although the overall sequence homology among these proteins is only 20-40% (Figure 34). In most class A enzymes such as CTX-M, Asp233 forms a short HB with Asp246. From the previous sub angstrom resolution x-ray crystallographic studies, this HB has a length of 2.47 Å and the hydrogen appears to be located equidistant to the two Asp Oδ atoms. 32 These observations suggest that this HB could be a low-barrier hydrogen bond (LBHB), a special type of short HB (~2.5 Å in length) where the hydrogen is equally shared between the two hydrogen bonding groups (for comparison, a typical HB is 2.8-3.2 Å). 41 Due to their special strength in the gas phase, LBHBs have been hypothesized to play an important role in proteins 39, 82, 149, 173, although their existence and functional relevance have been intensely debated 77, 98, 174-176, and some putative LBHBs have been found to be crystal artifacts 97. 177 For the Asp233-Asp246 HB, it is currently unknown whether it is indeed a LBHB, especially in solution. However, in other class A β-lactamase structures, the Asp233-Asp246 HB length remains short at approximately 2.5 Å. These enzymes include the clinically relevant class A enzyme KPC-2 (HB length 2.49 Å) 178 and another class A β-lactamase from a deep-sea bacteria (HB length 2.53 Å). 152 The short HB is even conserved in GES-2, where residue 233 is a glutamate, with a HB distance of 2.58 Å. 179 Interestingly, in a few enzymes where Asp246 is replaced by
isoleucine, another aspartate residue, Asp214 (Asn214 in CTX-M), forms an alternative short HB (~2.5 Å) with Asp233 (Figure 6). In the 0.90 Å resolution SHV-2 structure, a hydrogen atom is observed in the difference electron density map close to the center of the HB (2.59 Å in length) between these two residues.

The mutagenesis results suggest that the Asp233-Asp246 pair is important to the enzymatic activity of class A β-lactamases, achieved through maintaining the structural integrity of the active site involving loop 213-219 and neighboring residues. This function is particularly important for substrates with bulky side chains, such as cefotaxime, which can potentially destabilize or deform the active site. The special structural contribution of the Asp pair can be two-fold - fulfilling stringent electrostatic and steric requirements in a tightly packed region of the protein, and strengthening intramolecular interactions through a short, strong HB. My studies offer valuable insights into the first possible role of this Asp pair, highlighting the sensitivity of protein structures to the smallest change in residues. Because the Asp pair already shares a hydrogen atom between them, a single Asp->Asn mutation adds only one additional hydrogen to these residues in terms of the overall size, without changing the net charge of the pair. Yet, D246N failed to fold and express properly, and D233N exhibited significant structural distortions around the mutation. Although this is hardly surprising due to the well-known, densely packed, nature of protein interiors, it underscores the need for specific functional groups in particular regions of protein structure for optimal folding and function, a result of protein evolution. For that purpose, the Asp pair offers a unique piece of this 3-dimensional puzzle, as demonstrated by the WT and D233N structures. It is worth pointing out that, while the evidence for a O•••H-C interaction between Asp233Oδ2 and Gly217Hα is tenuous, Gly217 is also highly conserved in class A β-lactamases and the close contact between Asp233 and Gly217 is observed in other proteins as well. In addition, not all hydrogen-mediated carboxylate pairs are sensitive to mutations. For example, for the Glu15-Asp24
pair in the human protein DJ-1, substitutions of Gln15 and Asn24 are both well tolerated by the protein, albeit while decreasing the protein stability slightly.  

In comparison, my experiments do not offer direct answers to a possible special role of the short HB’s strength, particularly that of LBHB, which can be several times stronger than a standard HB. Previous studies have demonstrated that generally the shorter the HB length, the stronger its strength in non-aqueous solvent. It is hypothesized that the strength of a short HB can be beneficial for maintaining the active site integrity of class A β-lactamases. This is based on the high level of conservation of the Asp-Asp interaction in many class A β-lactamase structures that use Asp246 or alternatively Asp214 to form such a pair with Asp233. As previous experiments have shown, protein active sites, including those of β-lactamases, contain many high-energy features that only become complemented during substrate binding. Two such features in CTX-M include the sub-pocket recognizing the substrate C3(4)-carboxylate group, and the oxyanion hole. The carboxylate binding site is formed by the side chains of Thr235, Ser237, and Ser130 through direct HBs, as well as by Lys234 and Lys73 through long-range electrostatic interactions. In apo class A β-lactamase structures, this site is usually occupied by a phosphate ion or other negatively charged molecules from the crystallization buffer. The oxyanion hole is formed by the backbone amides of Ser70 and Ser237, the latter of which resides on the β3 strand. This subsite is crucial in stabilizing the transition state oxyanion, but the close juxtaposition of two partially positively charged amide groups is energetically unfavorable. In most apo structures, this sub-pocket is occupied by a water molecule. Interestingly, an Asp-Asp LBHB has been found close to the oxyanion hole of another unrelated enzyme, Rhamnogalacturonan acetylesterase, based on NMR analysis and the 2.47 Å short HB length in the crystal structure. In addition, a disulfide bond is also very often observed near the active site of many class A β-lactamase active sites, involving C69 and C238, located adjacent to the two residues making up the oxyanion hole.
Ser70 and residue 237. These studies suggest that the added strength of the Asp-Asp short HB could be important for countering the destabilizing internal interactions of the protein active site, particularly involving the oxyanion hole of β-lactamases.

**Protein flexibility in enzyme evolution and resistance development**

Structural flexibility plays a myriad of sometimes contrasting roles in protein function. While it confers adaptability and tunability of substrate recognition and enzymatic activity, the entropic cost can have an adverse effect on the ligand binding affinity, as well as the availability of productive active site configurations. Previous studies on β-lactamase evolution have demonstrated that increased flexibility in the active site can enable the enzyme to better accommodate larger substrates and broaden the spectrum of activity, and that these changes are often accompanied by additional mutations improving the overall stability of the protein or rigidifying local structures. The current results have shown that by increasing the conformational heterogeneity of certain structural features, β-lactamases can also drastically reduce the binding affinities of non-covalent inhibitors, while incurring only a modest cost on the activity for certain substrates (e.g., nitrocefin and ampicillin). The D233N mutation does not change the lowest-energy conformations of the residues in direct contact with the tetrazole-based inhibitor and many substrates, but rather results in a rise in the mobility of these active site residues, as partly indicated by the temperature factors of the crystal structure, as well as alternative conformations. Such effects can be more consequential for non-covalent inhibitors, specifically in changes to $k_{\text{off}}$, than for covalently bound substrates. However, for some substrates, such as cefotaxime, whose large side chains may cause deformation of the active site even in the WT enzyme and/or contact directly the regions distorted by the mutation, the negative impact on enzyme activity can nevertheless be significant.
Conclusion

Asp233 and Asp246 are highly conserved in class A β-lactamases. The mutagenesis and structural analysis has shed light on the contribution of this pair of aspartate residues to the integrity of the active site, particularly concerning the conformation of the 213-219 loop. The differential effects on the non-covalent inhibitor and various substrates suggest that β-lactamases can potentially develop resistance against certain inhibitors by increasing its active site flexibility. Furthermore, the Asp→Asn mutations illustrate the sensitivity of protein structure to the smallest change, i.e., the addition of one hydrogen atom. This has also made it difficult to isolate and quantify the effects of the D233N mutation on specific interactions. Nevertheless, I hypothesize that the short HB between Asp233 and Asp246 is likely stronger than a standard HB, and this strength may be important for maintaining the high-energy features of a protein active site. However, this particular role of the short HB will await future studies, including computational simulations.

Materials and Methods

Mutagenesis

CTX-M-14 (UniProt ID: H6UQI0) was cloned into a modified plasmid vector pET-9a as previously described. To make the CTX-M-14 mutants - D233N, T216A, and double mutant D233N/T216A - the QuikChange Lightning Site-Directed Mutagenesis Kit was used.

Protein purification, crystallization, and structure determination

The protein was purified as previously described and crystallized in 1.0-1.2 M potassium phosphate buffer (pH 8.3) from hanging drops at 20 °C. The final concentration of the protein in
the drop ranged from 6.5 mg/ml to 10 mg/ml. Full size crystals were grown in approximately two weeks with microseeding. The complex crystal of D233N and compound 1 was obtained by soaking the crystal in a solution of 10 mM compound for 12 hours prior to flash freezing with liquid nitrogen. For all apo and complex structures, a 30% w/v solution of sucrose was added to the crystallization mother liquor as a cryoprotectant. Diffraction data were collected on the SER-CAT 22-ID-D beamline at the Advanced Photon Source (APS), Argonne, Illinois. Data were indexed, scaled, and merged with HKL2000. The models for refinement were first obtained through using a rigid-body refinement by Phaser in PHENIX with an apo CTX-M-14 structure (PDB 4UA6). PHENIX and Coot were used to complete the model building and refinement. All figures were generated using PyMol (Schrodinger Inc).

**Biochemical assays**

The hydrolysis reaction kinetics of CTX-M and mutants were measured using the β-lactam substrates ampicillin and cefotaxime, as well as the chromogenic compound nitrocefin, in 100 mM Tris-HCl (pH 7.0) and monitored using a Biotek Synergy Mx monochromator-based multimode microplate reader at 235 nM, 260 nM, and 480 nM wavelengths respectively, at 37°C. Due to variation of enzyme activity and absorbance change based on the substrate, the concentration of enzyme used differed between different substrates to ensure the initial velocities of the reactions were accurately measured. The enzyme concentration was kept constant at 0.5 nM between assays for WT and mutants, where 10 nM for nitrocefin, 40 nM for ampicillin, and 100 nM for cefotaxime were used for compound concentrations. For inhibition analysis, nitrocefin was used as the substrate, and a concentration of 2 mM compound 1 was used as the highest concentration of inhibitor. $K_m$, $k_{cat}$ and $K_i$ were calculated using the software SigmaPlot.
**Thermal stability assays**

Using circular dichroism, the secondary structure was monitored with a Jasco J-815 CD spectropolarimeter in conjunction with a Petiltier cell holder. CTX-M-14 WT and D233N mutant were both diluted to 0.05, 0.1, and 0.2 mg/ml in 100 mM potassium phosphate buffer pH 7.0. All spectra were collected in triplicate for each of the three protein concentrations and measured using the wavelength 222 nm with a temperature range of 25 °C to 85 °C to determine the melting temperature. The collected data was analyzed using the software SigmaPlot and a two-state fitting program.

**Accession Codes**

The atomic coordinates and structure factors for the CTX-M-14 D233N, WT and complex structures have been deposited in the Protein Data Bank with IDs 6D7I, 6D7H and 7S0V, respectively. CTX-M-14 has Uniprot ID H6UQI0.

**Note to Reader #4**

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### Table 4. Biochemical characterization of CTX-M-14 β-lactamase and mutants

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* ND, not determined. Due to limited ligand quantities, $K_i$ values were averaged data sets of two trials.
Table 5. Asp-Asp X-Ray Data Collection and Refinement Statistics

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Refinement

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* Values in parentheses represent highest resolution shells
Figure 29. Chemical Structures of β-lactam Substrates and a Tetrazole-Based β-lactamase Inhibitor
Figure 30. Melting Temperature Analysis of CTX-M-14 WT and D233N Mutant.
Figure 31. Active Site of CTX-M-14 Class A β-Lactamase D233N Mutant vs WT Apo Active Site. 2Fo-Fc electron density map is shown in blue and contoured at 1.5 σ. Water molecules are shown as red spheres. Wat1 is the catalytic water. Potential HBs are represented by black dashed lines. A) CTX-M-14 WT (green) determined at 1.8 Å resolution in the P3₂1 space group. B) CTX-M-14 D233N mutant (cyan) determined to 2.0 Å resolution in the P3₂1 space group.
Figure 32. Conformational Changes Caused by D233N Mutation. Potential HBs in the mutant structure are represented by black dashed lines. A) Superimposed structures of CTX-M-14 WT (green) and D233N mutant (violet), highlighting the new interaction between the mutant Asn233 and backbone O atom of Gly217, and the shift in the 213-219 loop position. B) Superimposed active sites of WT and D233N mutant.
Figure 33. CTX-M-14 Class A β-lactamase D233N Mutant Compressed with Tetrazole Inhibitor 1. 2Fo-Fc electron density map is shown in blue and contoured at 1.5 σ. (A) CTX-M-14 D233N Mutant complexed with 1 determined to 1.95 Å in the P3_21 space group. (B) Superposition of D233N complex (cyan) with the mutant apo structure (magenta). (C) Superposition of WT (green) and mutant D233N complex structures.
Figure 34. Conservation of Asp-Asp Pairs in Class A β-lactamases. A) Apo structure of SHV-2 class A β-lactamase (PDB ID: 1N9B, magenta) superimposed with CTX-M-9 cefotaxime complex (PDB ID: 3HLW, green). Asp233 forms a short hydrogen bond with Asp246 CTXM-9, and with Asp214 in SHV-2. B) Sequence alignment of common Class A β-lactamases. Three positions for aspartate residues are highlighted. Asp233 is conserved in most Class A β-lactamases, with Asp246 also maintained in many of these enzymes. In a few proteins where Asp246 is replaced by isoleucine, Asp214 substitutes for Asn214. CTX-M-9 differs from CTX-M-14 only by a single V231A mutation.
Chapter 5:

Summary

The emergence of bacterial resistance seen in the clinical setting with the spread of newer, more efficient β-lactamase enzymes capable of hydrolyzing virtually all modern lactam antibiotics necessitates the need to have a deeper understanding of the underlying aspects of the β-lactamase hydrolysis mechanism. My work presented here follows the logical course from β-lactamase function to inhibition and finally resistance. Within the second chapter I focused on the functional analysis of the class A β-lactamase mechanism using a wide array of techniques to resolve individual states of the mechanistic pathway. Serine β-lactamase enzymes are hydrolases and are governed by the shuffling of protons in the active site between catalytic residues during different stages of the mechanism. If we can unequivocally resolve protonation states of specific stages of the mechanism, we can design better, more potent inhibitors. I started with a proof-of-concept project proving I was able to generate a neutron diffraction quality crystal for a data set of the CTX-M-9 apo enzyme, where all protonation states of the key catalytic residues were resolved. The next structure I presented was of the perdeuterated CTX-M-9 structure in complex with the tetrazole based inhibitor 3GK that traps the mechanism in the pre-covalent state. The structure is one of the highest resolution neutron structures compared to deposited structures in the protein data bank and shows all of the protonation states in the active site. There is ambiguity surrounding the direction of the Ser70 proton, but Lysy73 and Glu166 are both clearly neutral with a bond distance associated with a potential LBHB between Ser70 and Lys73. The third structure presented in the chapter was of the diacylation transition state through the boronic acid
transition state analogue SM23. The sub angstrom resolution x-ray structure was resolved to a resolution of 0.76 Å and clearly designates a tetrahedral intermediate with a positively charged Lys73 and a neutral Glu166. The structure shows an interesting bond distance between the residue Glu166 and a boronyl oxygen atom at a distance of 2.47 Å with a partially delocalized hydrogen in the Fo-Fc map. This could potentially be an instance of a LBHB, but needs further verification through either the use of NMR or QM/MM calculations. The last set of structures from this chapter include a progression where I show a selective mutational study of instilling a disulfide bond into the active site through point mutation of Glu166Cys and Asn170Aala. The potential for this system to selectively instill a perturbed microenvironment has many utilities to investigate the previously seen and verified LBHB between Ser70 and Lys73 in the pre-covalent complex.

The third chapter concerned inhibition of serine β-lactamase through the use of CTX-M-14 and CTX-M-27 as model systems for the investigation of amide-heteroarene pi stacking interactions. I generated five complex crystal structures with tetrazole-based scaffolds that all showed good promise to be used as a models as all of the structures adopted the expected binding pose with the heteroarene stacking on the amide of Gly238 of the β3 strand. The obtained structures were of a resolution range that could not unambiguously define rotomeric states, and thus future work entails obtaining a higher resolution, sub angstrom crystal structure in order to fully define the state un unsymmetric heteroarene substituents.

The fourth chapter entailed the study of resistance mechanism through the analysis of aspartate – aspartate interactions in CTX-M-14. I obtained three crystal structures investigating the interaction between Asp233-Asp246, a WT structure, the mutant D233N structure complexed with the tetrazole-based scaffold J1X. This work shed light on the contribution of this pair of aspartate residues to the integrity of the active site, particularly concerning the conformation of
the 213-219 loop. My findings suggest that β-lactamases can potentially develop resistance against certain inhibitors by increasing its active site flexibility.
Chapter 6:

References Cited


Improving the accuracy and resolution of neutron crystallographic data by three-dimensional profile fitting of Bragg peaks in reciprocal space, *Acta Crystallographica Section D* 74.


Appendix 1:

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