RAMAN SPECTROSCOPIC STUDIES OF INHIBITOR REACTIONS IN CLASS A, B AND D β-LACTAMASES

by

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proprietary material contained therein.
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### List of Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>B3LYP</td>
<td>Becke, three-parameter, Lee-Yang-Parr</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged-coupled device</td>
</tr>
<tr>
<td>DHF</td>
<td>Dihydrofolic acid</td>
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<tr>
<td>DFT</td>
<td>Density functional theory</td>
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<td>SHV glutamate166alanine variant</td>
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Raman Spectroscopic Studies of Inhibitor Reactions in Class A, B and D β-Lactamases

Abstract

by

TAO CHE

The driving force behind my research is the need to develop improved antibiotic therapies to combat the drug resistance. The production of β-lactamase is the major cause of failure of β-lactam-based therapy against Gram-negative bacteria, such as *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. However, currently there are only three β-lactamase inhibitors in clinical use and they narrowly target the class A enzymes. A clinically useful inhibitor needs to have a significantly broader inhibitory spectrum, ideally targeting all serine β-lactamases (class A, C and D).

My work is focused on studying the catalytic mechanisms of serine β-lactamases, and thence aiding in the design new mechanism-based inhibitors. A major thrust involves use of Raman spectroscopy which is a laser light scattering technique that provides structural information of the bound ligand in the active site of the enzymes in real time. With the combination of Raman spectroscopy and other structural or microbiological methodologies, we aim to address two fundamental questions in drug discovery: 1) why is a compound a good inhibitor for a target enzyme *in vitro*? 2) how effectively is a drug taken up by cells?
We have designed and implemented a new method by combining rapid mix-rapid freeze of aqueous solution with Raman spectroscopy. This enables us to trap intermediates early on the reaction pathway- within milliseconds.

By studying the reaction of OXA-24 and OXA-1 and a penicillin derivative inhibitor in *crystallo* and in solution, we have shown that carboxylation of the lysine is critical for class D enzymes' catalysis, while decarboxylation can be induced by inhibitors and therefore, leading to enzyme inactivation.

Penetration barriers and efflux pumps are two major mechanisms bacteria adopt to exclude drug molecules. The difficulty in directly measuring compound penetration is a major reason for the lack of progress in antibiotic discovery. Using a new protocol, the intermediates or products formed inside the bacterial cells have been captured, which allows us to investigate reactions inside bacterial cells.
Chapter I: Introduction

The battle between bacteria and the human population never seems to end. Before the discovery of the first antibiotic penicillin, millions of patients lost their lives due to bacterial infections. The introduction of penicillin during the World War II is definitely one of the most successful forms of chemotherapy in the twentieth century. With the advent of the penicillin, the golden era of antibiotic discovery began and lasted for more than 20 years, from 1940s to 1960s. Almost all antibiotics in use today were screened from soil-dwelling Actinomycetes during that time (Figure I.1) (1).

![Timeline of clinical antibiotic development](image)

**Figure I. 1** Timeline of clinical antibiotic development

The targets of these antibiotics are versatile (Figure I.2). They are all involved in key events during the bacterial life cycle. These events are unique in bacteria so that there are few side effects on eukaryotes or even humans. For example, penicillin, as well as other β-lactams (cephalosporins, carbapenems, monobactam etc), bind to the cell wall synthesizing enzymes, preventing the latter using peptidoglycan to form a larger cell wall network. Streptomycin, the first aminoglycoside, inhibits bacterial protein synthesis by specifically binding to 16S ribosomal RNA. Ciprofloxacin, a quinolone antibiotic, targets
the DNA topoisomerases and causes DNA breaks and replication fork arrest. The above-mentioned three classes are also perceived as the most successful antibiotics. They have unique mechanisms of killing by modulating their respective targets. However, the golden era of antibiotic discovery is over. The pace of discovery of new antibiotics from the early 1960s to the present days has dramatically slowed (2). One of the reasons for few antibiotics synthesized or discovered in the past 50 years is because, during this time the issue of antibiotic resistance was becoming increasingly problematic. However, very recently Ling et al. isolated a new antibiotic, teixobactin, from uncultured bacteria and the new antibiotic kills bacteria without inducing resistance (3). Perhaps this will shift our attention toward those untapped source in the external environment.

Figure I. 2 The targets of clinically used antibiotics in bacterial cell.
Antibiotic resistance is now a well-known phenomenon and seems to be unpreventable. Simply as its name implies, resistance means the continued growth of bacterial pathogens under the bioavailable concentration of antibiotic. The development and spread of antibiotic resistance has put great pressure on both science and medicine. It causes serious clinical failures because the pathogens of some severe infections are resistant to almost all currently available drugs (4). It is reported that the economic cost associated with these infections exceeds $20 billion/year in the US (5). More effective ways are sorely needed to tackle antibiotic resistance. Although antibiotic resistance cannot be eradicated, it can nevertheless be controlled.

Antibiotic resistance is not a modern event. Soon after penicillin was first introduced into the market in 1942, resistant bacterial strains were isolated in 1948. Part of the research community claimed afterwards that antibiotic resistance is induced by the broad application of antibiotics. However, recently D'Costa et al. (6) reported that antibiotic resistance is ancient based on DNA sequence analysis. It is a natural phenomenon that resistant genes are embedded in the bacterial genome before the use of clinical antibiotic. Those bacteria are distributed everywhere in humans, animals and natural environments such as soil and water. The problem is what kind of conditions or factors lead to activation and transmission of these resistant genes among species. We still do not have sufficient knowledge and a good platform to predict when and where the resistance emerges. What is the mechanism underlying the activation of resistant genes and expression of relevant proteins? The Lewis group pointed out that the dormant persister bacterial cells play an important role as a potential reservoir of antibiotic resistant bacteria (7). Are those persisters acting like the bacterial "stem cells"?
Although the use of antibiotics does not directly "create" the resistant bacteria, it is definitely accelerating the mobilization and selection of the resistant genes. The abuse of antibiotics in animal feed, agriculture and fisheries to promote growth takes up more than 70% of the total usage annually in the US. Another problem is that we now have many more available compounds than currently exploited targets. That means, we have a large reservoir of antibiotics discovered from nature or synthesized through chemical modifications, but the number of their targets is really small. Most of them are restricted to reacting with penicillin binding proteins (cell wall synthesis), ribosomes (protein synthesis) and DNA topoisomerases (DNA replication). There are about 200 conserved essential proteins present in bacterial cells but less than 10% have been used as potential targets (1). The lack of cell penetrating ability is a common deficiency in those newly discovered or designed compounds. How to overcome this effective barrier in bacteria is a key problem to be addressed.

Meanwhile, bacteria are always growing and dividing. Bacterial evolution is overwhelming current antibiotic therapy and new resistant cell types have evolved with specific strategies to overcome the antibiotics (Figure I.3) (8). For instance, to combat the β-lactams that target cell wall synthesis, bacteria produce an enzyme, β-lactamase, to specifically hydrolyze antibiotics; for those antibiotics functioning on DNA topoisomerase or ribosome, bacteria introduce modifications or mutations in their sub-domains to decrease the affinity of the drugs; for other antibiotic drugs, bacteria cells either overexpress efflux pumps to eject the drugs out of the cells, or decrease the amount of porins in the membrane to restrict the drug entrance. In this thesis, we focus on the
The β-lactams make up one of the three largest antibiotic classes (the other two are macrolides and quinolones). The β-lactam family includes natural and synthetic compounds such as penicillins, cephalosporins, carbapenems and monobactams. They all interfere with cell wall synthesis and narrowly target the penicillin binding proteins (PBP). Cell wall synthesis is a unique event in Gram-negative bacteria, therefore, it is an ideal target to avoid side effects on host cells. The bacterial cell wall consists of peptidoglycan units that help maintain the cell shape and preserve osmotic stability. The
peptidoglycan unit is first synthesized in the cytoplasm through several catalytic steps and is then flipped to the external side of the cell membrane, where PBPs catalyze transglycosylation and transpeptidation reactions (9). Thus, PBPs are involved in the final stage of the synthesis of peptidoglycan, which is the major component of bacterial cell wall. Its name, penicillin binding protein, just reflects the way by which the protein was discovered. It catalyzes the crosslinking via two steps (Figure I.4): the first is the removal of the D-Alanine from the precursor peptidoglycan; the second is the catalysis of peptide bond formation with its transpeptidation function. As a result, the newly synthesized glycan strands are polymerized and crosslinked via flexible peptides, forming a rigid peptidoglycan network.

**Figure I. 4** PBP-catalyzed peptidoglycan crosslinking.
In the active site of PBPs, there is a serine that attacks the carbonyl group (Figure I.5), releases the alanine and forms the trans-peptide bond. That serine at the active site of PBPs is conserved in all members of PBP family. The reason for why penicillin has high affinity with PBPs and is effective against PBPs, is because penicillin is similar in chemical structure to the modular pieces that forms the peptidoglycan. When penicillin binds to PBPs, the active site serine attacks the carbonyl group in penicillin (Figure I.5) and forms a covalent bond with the catalytic serine, which is an essentially irreversible reaction and inactivates the PBPs.

**Figure I. 5** Comparison of catalytic mechanisms between PBPs and β-lactamases.

However, due to the selective pressure, bacteria also adopt defensive strategies to overcome β-lactam antibiotics (Figure I.3). i) **Decreased expression of porin channels in outer membrane.** For example, the loss of OprD in the outer membrane plays a role in imipenem and meropenem resistance in *Pseudomonas aeruginosa* (10); multidrug-
resistance in *Acinetobacter baumannii*, a clinically important isolate, is partially attributed to the loss of CarO porins (11). **ii) Overexpression of efflux pumps in the outer membrane.** They are capable of exporting β-lactam antibiotics from the periplasm to the surrounding environment, reducing the β-lactam concentration from cytotoxic to acceptable. Studies have shown that upregulation of AdeABC efflux pump in *A. baumannii* contributes the dramatic increase in carbapenem MIC (minimum inhibitory concentration) (12, 13). **iii) Mutations in the active site of PBPs.** Mutations contribute to the resistance by lowering the affinity for β-lactam antibiotics and PBPs. PBP2a, found in methicillin-resistant *Staphylococcus aureus*, plays a major role in the latter's broad clinical resistance to β-lactam antibiotics (14). Interestingly, the β-lactam resistance mediated by PBPs are more commonly found in Gram-positive than in Gram-negative bacteria. For example, there have been no reports of altered PBPs associated with β-lactam resistance in *Escherichia coli* so far. This is probably due to the fourth resistant mechanism, **iv) production of effective β-lactamases.** This is also the focus of the rest of my thesis. β-lactamase enzymes are the most common and effective approach by Gram-negative bacteria to get rid of β-lactams. Most β-lactamases also have a catalytic serine in the active site like PBPs. This serine attacks the carbonyl group of the β-lactam ring and then forms the acyl-enzyme complex. Differing from PBPs, β-lactamases can utilize a water molecule to specifically hydrolyze the acyl-enzyme complex. Nowadays, it is generally accepted that β-lactamases might have evolved from PBPs because they are very similar in sequence, structures and catalytic mechanisms (Figure I.5) (15, 16). Before going into the details of β-lactamase, it is worthwhile to point out that above mentioned resistance mechanisms do not work alone, typically they function in
combination with β-lactamase expression. In present day clinical trials, some Gram-negative bacterial infections are usually treated with two drugs: one such as penicillin to inhibit the cell wall synthesis, the other one such as inhibitors to inactivate the β-lactamases thereby blocking penicillin hydrolysis (Figure I.6).

![Diagram of bacterial cell wall and β-lactamase inhibition](image)

**Figure I.6** The working model of combinational therapy in the clinic: β-lactams and β-lactamase inhibitors.

### I-2 β-Lactamases

β-Lactamase production is the most important mechanism by which Gram-negative bacteria become resistant to β-lactam antibiotics. To date, more than 1600 unique β-lactamases have been reported (17). Based on their protein sequence similarities, they are divided into four classes (Class A-D, Figure I.7) (18). In Figure I.7, class A, C and D β-lactamases have similar structures. This is consistent with their function. They share a common mechanism that uses an active-site serine to attack the antibiotics. Class B β-lactamases are special and are zinc-dependent. The 1600 β-lactamases are distributed in different bacteria which has put our defense against bacteria at risk.
Figure I. 7 “Family portrait” of β-lactamase enzymes. (A) Class A SHV-1; (B) Class B NDM-1; (C) Class C AmpC; (D) Class D OXA-1. For class B, the two Zn ions are shown in orange. (Based on PDB entries 1SHV, 3SPU, 2BLS, and 1M6K, respectively.)

The reaction mechanism of β-lactamase with a β-lactam antibiotic can be depicted in Scheme I.1. In Scheme I.1, E is a β-lactamase, S is a substrate, such as penicillin, E:S is the Michaelis-Menten complex, E-S is the acyl enzyme complex, and P is the hydrolyzed product. After the reaction, the β-lactamase is regenerated and able to hydrolyze the next β-lactam antibiotic again and again.
Scheme 1. General scheme for enzyme-substrate reactions.

\[
E + S \xrightleftharpoons[k_{-1}]{k_1} E : S \xrightarrow{k_2} E -- S \xrightarrow[k_3]{H_2O} E + P
\]

M-M complex   Acyl-enzyme   Regeneration of active enzyme

**Class A \(\beta\)-lactamases.** The Ambler class A is the largest serine \(\beta\)-lactamase class. Interestingly, the class A \(\beta\)-lactamase formerly is also evolutionarily related to PBPs. Sequence analysis shows that PBP4 and PBP5 in *E. coli* displays high similarity to class A enzymes (19). The catalytic motif Ser-x-x-Lys is conserved in both PBPs and serine \(\beta\)-lactamase. This class of enzymes was previously described as "penicillinases" because their affinity with penicillin and efficiency to inactivate penicillin was much higher than for other \(\beta\)-lactam antibiotics. The catalytic rate constant \(k_{cat}/K_m\) reaches the diffusion limit under certain conditions. The first plasmid-mediated \(\beta\)-lactamase, TEM-1, was identified in *E. coli* in 1963 and also belongs to the class A \(\beta\)-lactamases. Another common class A \(\beta\)-lactamase SHV-1, found in *K. pneumoniae*, shares 68% sequence homology with TEM-1. Since then, many more homologous \(\beta\)-lactamases associated with TEM-1 or SHV-1 carrying a single or multiple amino acid mutations have been identified. Today these two subclasses are the most abundant species among \(\beta\)-lactamases with 135 TEM and 57 SHV in 2004 and 219 TEM and 185 SHV in 2014 (http://www.lahey.org/studies/temtable.asp). Based on the substrate profiles, class A \(\beta\)-lactamases can be divided into three subgroups (Table I.1). They are responsible for the resistance in many identified clinical pathogens. For example, the selective pressure from the expansive use of antibiotic leads to the emergence of the extended-spectrum \(\beta\)-
lactamases (ESBLs) and carbapenemases, such as CTX-Ms and KPCs, respectively. They can hydrolyze the new generation antibiotics like cephalosporins and monobactam aztreonam which are considered as the last resort against β-lactamase-mediated resistance.

**Table 1.1** Classifications of class A β-lactamases.

<table>
<thead>
<tr>
<th>Class A β-lactamase</th>
<th>Substrate types</th>
<th>Enzymes included</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillinases</td>
<td>penicillin, narrow-spectrum cephalosporins</td>
<td>TEM-1, TEM-2, SHV-1</td>
</tr>
<tr>
<td>ESBLs</td>
<td>extended-spectrum cephalosporins</td>
<td>SHV-2, TEM-3, CTX-Ms</td>
</tr>
<tr>
<td>Carbapenemases</td>
<td>cephalosporins, carbapenems</td>
<td>KPC-2, NMC-A</td>
</tr>
</tbody>
</table>

In the clinic, to inhibit class A β-lactamases, commercially available β-lactamase inhibitors (sulbactam, tazobactam and clavulanate, Figure 1.8) have been applied in combination with β-lactam antibiotics (Amoxicillin-clavulanate, Ticarcillin-calvulanate, Ampicillin-sulbactam and Piperacillin-tazobactam). They have a common scaffold (a β-lactam ring fused with a five-membered ring) in common with penicillin to ensure high affinity with β-lactamases. But it is noteworthy that these inhibitors alone do have intrinsic antibiotic activity, such as sulbactam against *Bacteroides spp.*, *Acinetobacter spp*; clavulanate against *Haemophilus influenzae*; tazobactam inhibition of PBPs in *Borrelia burgdorferi* (19). However, the activity is weak and also reduced by penetration barrier issues and β-lactamase hydrolysis; this is why, for clinical use, they are combined with β-lactam antibiotics.
The mechanisms of inhibition by these clinical inhibitors have been well characterized, as shown in Scheme I.2. This generally accepted reaction mechanism for suicide-type inhibitor, tazobactam, also provides the background for experiments our lab undertook involving sulfone penams. It is believed that the serine-based enzymes form Michaelis-type, non-bonded complexes with the inhibitors, which resembles the natural substrates for β-lactamases. However, this is followed by nucleophilic attack at the lactam carbonyl by a serine side-chain. This leads to the formation of a transiently inhibited enzyme in which imine acyl-enzyme tautomerizes to yield the more stable cis- or trans-enamine-based (Scheme I.2) structures that have been associated with an absorption band detected at 280 nm (20-22). A second possible pathway is where a second enzymatic nucleophile, likely Ser130 in the class A family, attacks the C5 position of the reactive imine acyl-enzyme, leading to β-elimination across the C5-C6
bond and formation of a 5-atom vinyl carboxylic acid moiety attached to the second nucleophile (Ser-acrylate, Scheme I.2).

Although class A β-lactamases are generally susceptible to clinical inhibitors, more and more exceptions have been quickly discovered and identified due to mutations found in the ESBLs and carbapenemases. What makes it worse is that these clinical inhibitors (sulbactam, tazobactam and clavulanate) narrowly target class A β-lactamases, they have little or no inhibition against class B, C and D β-lactamases. A growing number of bacterial strains are identified expressing more than one type of β-lactamase as a defense. Thus, ideal inhibitors with a broader spectrum of inhibition are urgently needed. The mechanism by which Gram-negative bacteria acquire multiple β-lactamases or β-lactamases with broad substrate specificity such as KPCs or MBLs remains a rich area of research.

**Scheme I. 2** General mechanisms for clinical inhibitors reacting with class A β-lactamases (tazobactam shown, similar mechanism for sulbactam and clavulanate).
**Class B metallo-β-lactamase.** Class B β-lactamases are metallo-enzymes that require one or two zins to catalyze hydrolysis of the lactam ring. Due to this unique feature, class B β-lactamases are becoming one of the biggest challenges in the clinic because they can hydrolyze almost all the β-lactams and they are not susceptible to clinically used β-lactamases inhibitors, such as sulbactam and tazobactam. The only exception is the monobactam, aztreonam, which is proposed to be a poor substrate of class B β-lactamases.

Based on DNA sequence similarity, class B β-lactamases are classified into three subgroups, B1, B2 and B3. The B1 family shares 23% sequence identity among each other and this family includes the first discovered class B β-lactamase BcII from *Bacillus cereus* (23), CcrA from *Bacteroides fragilis* (24) and recently isolated NDM-1 from *K. pneumoniae* and *E. coli* (25). The B2 family shows only 11% identity among its members and includes the most studied CphA from *Aeromonas hydrophila* (26), ImiS from *Aeromonas veronii* (27). The B3 enzymes have only nine conserved residues and this subclass includes L1 from *Xanthomonas maltophilia* (28), GOB-1 from *Chryseobacterium meningosepticum* (29).

Although these metallo enzymes have relatively low sequence conservations, they all adopt a similar folding, αββα, based on their available crystallographic structures. This is consistent with their catalytic mechanism that incorporates one or two zinc ions in the active site to initiate the nucleophilic attack (30). Particularly, B1 and B3 enzymes are proposed to utilize a binuclear center in the catalytic reactions (Scheme I.3). Take B1 NDM-1 enzyme for an instance (PDB ID: 3SPU) (31), Zn1 is coordinated with His120, His122, His189 and a catalytic water molecule. This zinc ion acts as a Lewis acid to
decrease the pK$_a$ of the water molecule to 5-6, resulting in the deprotonation of the catalytic water molecule at neutral pH. Thus the deprotonated water molecule, a hydroxide, can attack the carbonyl group of the β-lactam ring. Zn2 coordinates with Asp120, Cys208 and His250 and adopts a tetrahedral geometry, similar conformation as for Zn1. It is proposed that the Zn2 helps the deprotonation of the catalytic water molecule, as well as the substrate binding and stabilization of the intermediate after opening of the β-lactam ring. Thus, the two zinc ions in B1 or B3 enzymes are not functionally equivalent. Weak catalytic efficiency is still maintained when B1 or B3 enzymes are in the single zinc form, but is optimal in the bimetallo form.

**Scheme I.** Schematic representation of the Zn binding sites in dinuclear class B enzymes.

The subclass B2 enzymes utilize a monozinc mechanism where the zinc is equivalent in position and function to Zn2 of the B1 and B3 enzymes (32). This zinc acts as a Lewis acid and abstracts a proton from a water molecule with the resulting OH$^-$ attacking the carbonyl group in the β-lactam ring. However, a different reaction mechanism has also been proposed where Asp120 in the active site acts as a general base
to deprotonate the catalytic water molecule because the monozinc is found not directly interacting with a nucleophilic water molecule (33).

Although there are three serine-β-lactamase inhibitors in the clinic and several others in clinical trials, all of them are inefficient towards class B β-lactamases. Inactivators tried early such as chelators EDTA, dipicolinic acid and 10-phenanthroline showed no clinical significance (34). King et al. recently reviewed the literature on inhibitors of class B β-lactamases (30), which include penicillin-, celphalosorin- and carbapenem derivatives. These substrate analogs still represent the most promising approach to tackle class B β-lactamases because they show high affinity with the enzyme. Other examples such as thiol derivatives succinic acid and pyridine carboxylates are also the candidate as chelators to inactivate the zinc ions. However, these compounds are narrow-spectrum inhibitors that only show efficacy against one or two class B enzymes in biochemical and microbiological assays. In 2014, King et al. identified that a natural product originally isolated from Aspergillus versicolor, aspergillomarasmine A, which shows potent inhibitory effects towards NDM-1 and VIM-2 both in vitro and in vivo. This is therefore an excellent lead for compounds for the clinical treatment of class B β-lactamase mediated infectious diseases (35).

**Class D β-lactamases.** Unlike class A and C enzymes, which have been extensively studied, less information is available for class D β-lactamases. The class D β-lactamases are characterized by the presence of a carboxylated lysine in the active site that participates in catalysis. Class D β-lactamase, also known as oxacillinases or OXAs, due to their enhanced capability to hydrolyze oxacillin, can also possess carbapenemase or ESBL (Extended Spectrum Beta-Lactamase) activity. They are important determinants
of antimicrobial resistance in Gram-negative pathogens, particularly including *A. baumannii* and *P. aeruginosa* (36, 37). Class D enzymes are frequently not inhibited by the commercial β-lactamase inhibitors, clavulanic acid, sulbactam, and tazobactam. The class D β-lactamases have a characteristic hydrolytic mechanism involving a carboxylated lysine residue, which is believed to function as the catalytic base, during both initial acylation of the active site serine and hydrolysis of the intermediate acyl-enzyme (38), as shown in Scheme I.4 (39). However, the carboxylation is reversible. That means, under certain condition, such as low pH, the lysine can be decarboxylated. While there are examples of carboxylated lysine residues in other enzymes, it is unusual for this residue to have a direct mechanistic role.

**Scheme I. 4** Proposed mechanism for class D enzymes catalyzed hydrolysis.

In the OXA family, the structural basis of the substrate specificity is understood only at a rudimentary level. One of the most significant multi-city outbreaks of infections or colonization due to high-level imipenem-resistant *A. baumannii* was caused by OXA-24 (Figure I.9) (40). One hundred isolates in the Chicago area were
from diverse sources and were obtained from seven acute-care hospitals and two extended-care facilities; 97% of the isolates belonged to one clone. This is one of the largest outbreaks in the US. This emerging problem has also been described in Spain (41, 42) and Portugal (43). Most significantly, β-lactamase inhibitor studies showed that OXA-24 is weakly inhibited by clavulanic acid ($K_i = 300 \mu$M), tazobactam (180 µM), and sulbactam (190 µM) (44). The widespread dissemination of OXA-24 underscores its importance. At present, there are very few carbapenem-hydrolyzing class D enzyme (OXA-24 and OXA-48) crystal structures available for study (45).

**Figure 1. 9** Active site of OXA-24 class D β-lactamase. S81, nucleophile; Carboxylated lysine K84 is shown in red circle.

**I-3 Raman techniques for following enzyme-inhibitor reactions**

**Raman spectroscopy---reactions in crystals.** For a decade, our laboratory has developed Raman crystallography to track reactions of clinical inhibitors in single
crystals of β-lactamase. A Raman microscope is used to acquire the Raman spectrum of a single protein crystal. The method is set out in Figure 1.10 (46) for a single protein crystal held in a hanging drop under conditions similar to those used for growing crystals (47). After the substrate is injected into the holding solution which contains the protein crystal, the reaction occurs. Any change due to the enzyme-substrate reaction in the crystals will be recorded in the scattered light. By analyzing the scattered light we can get the information on the intermediate or product. The subtraction (Raman spectrum complex) minus (Raman spectrum free enzyme) is carried out using a computer. This yields the Raman difference spectrum of the bound ligand, although this may be less than 1% of the total signal prior to subtraction (48, 49). It is the Raman difference spectrum that provides us useful information on the chemical changes of the substrate.

An important reason of using Raman crystallography is that direct measurement of reactions in aqueous solution is difficult because the Raman signal is too weak to be captured. However, we found that many of the problems encountered in solution work essentially disappear when the complexes are studied in single protein crystals (50-52). The single crystal approach works best if the ligand can be “soaked in” and “soaked out”. When this condition is met, superb quality Raman difference spectra are usually obtained (53). One limitation is that it does not allow to study the early process in drug-enzyme recognition because the average time for collecting one spectrum is about 2 min.
**Figure I. 10** Raman set-up for following enzyme-substrate reactions in crystals.

**Raman spectroscopy---reactions in solution.** A more general method, applicable to a wide range of time scales, is rapid mixing - rapid flow (Figure I.11), followed by freeze quenching to trap intermediates on the reaction pathway. In many ways early events on the reaction pathway represent the key step of β-lactamase reactions. Although the initial encounter complexes, and short-lived intermediates as they interconvert, may be vital in determining the formation of quasi-stable species, there is presently little or no molecular detail at short timescales. Time resolution in crystal studies is presently >1 minute. Experiments in solution have the potential to obtain data on shorter time scales but only produce weak Raman signal. Consequently, we have adapted rapid-freeze - rapid freeze technology to trap solution-generated complexes. Using a rapid mixing instrument in our lab, enzyme and inhibitor solutions are put into two syringes connected to a mixing jet where they are mixed and then frozen by running them into a low temperature bath. The intermediate population is trapped in the "ice"
generated in the isopentane surrounded by a bath of liquid nitrogen. The enzyme and substrate mixture enters into the isopentane to immediately form an ice sample containing a frozen intermediate. The ice is then freeze-dried and the sample is characterized by Raman microscopy.

![Schematic of rapid mix - rapid freeze system.](image)

**Figure I. 11** Schematic of rapid mix - rapid freeze system.

We have reported the combination of rapid mixing - rapid freezing, and freeze-drying, and analyzing the freeze-dried powder by Raman microscopy to investigate an enzyme-substrate reaction (54). The sensitivity of Raman signal is enhanced by freeze-drying the sample. The rapid mixing - rapid freezing results for tazobactam reacting with SHV-1 show analogous intermediates compared to those in crystals. However, the lifetimes, in relative and absolute terms, are very different. The results confirm the presence of a stable trans-enamine in solution and this is the species that can be used for strategy-based drug design (55). The enzyme properties are not changed after the freeze
conditions (Figure I.12). The catalytic efficiency towards the substrate nitrocefin remains nearly the same before and after freeze-drying.

![Graph](image)

**Figure I. 12** Comparison of enzymatic activity of SHV-1 β-lactamase reacting with nitrocefin before and after lyophilization. 1 unreacted SHV-1 (black solid line), 2 freeze-dried SHV-1 (black dash line), 3 untreated SHV-1 with 1 µM tazobactam (blue solid line), 4 freeze-dried SHV-1 with 1 µM tazobactam (blue dash line), 5 untreated SHV-1 with 10 µM tazobactam (green solid line), and 6 freeze-dried SHV-1 with 10 µM tazobactam (green dash line).

**Raman spectroscopy---reactions in bacterial cells.** In earlier *in vitro* studies (46, 47, 53, 56-66) we have uncovered a plethora of complex reaction schemes for mechanism-based β-lactamase inhibition. Using a new protocol recently developed in our lab, we can quantitate the number of drug molecules in each cell and characterize the drug-target reactions *in vivo*. This is obviously an important issue since drug design depends on knowing which species is blocking the active site. Two related protocols have been developed to measure drug populations in cells. In the initial work (67) cells were
grown in the presence of a known amount of drug, harvested, freeze dried and analyzed by Raman microscopy. In a more recent approach live cells are transferred from medium to a holding buffer containing the compound. The advantages of the latter method are that the Raman difference spectra have fewer interfering contributions originating from changes in cell metabolism that occur during growth cycle in the presence of the drug, and, in addition the soak in time is up to 1 hour whereas soaking in to growing cells requires 2-6 hours, depending on the strain.

The detailed protocol is shown in Figure I.13. Briefly speaking, bacterial cells are incubated with certain amounts of drugs for variable time period. Then the drug molecules that are outside the cells are removed by spinning down the cells and repeated washing. The cell pellets are collected and freeze dried. The dried sample is characterized by Raman microscopy. One advantage of this protocol is we can quantitate drug population inside the cells. This may help us explain any discrepancies between IC$_{50}$s and MICs. If the sample library is big enough, we can even create rules for effective drug penetration; the other advantage is that the protocol allows us to obtain the conformational information on drug-target complexes within the cellular environment. By comparing this with the results from analogous in vitro reactions, we can compare the reaction mechanisms in vitro and in vivo.
Figure I. 13 Protocol for following drug-target reactions inside bacterial cells.
Chapter II: Raman Spectra of Interchanging β-Lactamase Inhibitor Intermediates on the Millisecond Time Scale

ABSTRACT: Rapid mix - rapid freeze is a powerful method to study the mechanisms of enzyme-substrate reactions in solution. Here we report a protocol that combines this method with normal (non-resonance) Raman microscopy to enable us to define molecular details of intermediates at early time points. With this combined method, SHV-1, a class A β-lactamase, and tazobactam, a commercially available β-lactamase inhibitor, were rapidly mixed on the millisecond time-scale, then were flash-frozen by injecting into an isopentane solution surrounded by liquid nitrogen. The "ice" was finally freeze-dried and characterized by Raman microscopy. We found that, in solution at 25 milliseconds, the reaction is almost complete giving rise to a major population composed of the trans-enamine intermediate. Between 25 - 500 milliseconds, minor populations of protonated imine are detected, that have previously been postulated to precede enamine intermediates. However, within 1 second, the imines are converted entirely to enamines. Interestingly, with this method, we can measure directly the turnover number of SHV-1 and tazobactam. At 1 : 4 ratio (enzyme : inhibitor) or greater, the enzyme is completely inhibited, a number that agrees with the turnover number derived from steady-state kinetic methods. This application, employing non-intensity enhanced Raman spectroscopy, provides a general and effective route to study the early events in enzyme-substrate reactions.

This chapter has been published in the Journal of American Chemical Society and has been reproduced in accordance with the approval. J Am Chem Soc, (2013), 135, 2895-2898.
II.1 INTRODUCTION

In general, there are two ways to detect the early intermediates in an enzyme-substrate reaction: slow the reaction or trap the intermediate. By lowering reaction temperature, one can reduce the reaction rate to a detectable time range (68, 69). Another approach is to switch on the reaction by the excitation from a pulse of light and then probe the intermediate with ultrafast spectroscopic tools (70, 71). These techniques, however, are limited to specific reaction systems. With the advent of the rapid mixing - rapid quench technique the pace of studying meta-stable reaction intermediates accelerated significantly. The continuous improvement on mixers has made this technique an attractive tool because of its short time scales and low sample consumption (72-76). The freeze-quenching technique was first introduced by Bray in 1961 where the mixed reactant solution is rapidly frozen by low-temperature isopentane (-130 °C) (77).

Raman or resonance Raman spectra of reaction intermediates in water have the potential of providing a plethora of information on enzyme mechanism (47, 78, 79). In 2003 Lin et al. combined ultrafast mixing with a rapid freeze-quenching device and could detect intermediates as early as 50 microseconds (80). The resulting ice containing the reaction intermediates gave high quality resonance Raman spectra. However, the intensities of normal (non-resonance) Raman spectra of biological molecules at millimolar concentrations are usually too weak to provide sufficient signal-to-noise data for analysis and this limitation remains when the intermediates are trapped in ice. We now report a protocol which allows us to obtain time-resolved normal Raman spectra of aqueous intermediates in the millisecond time scale. Rapid mix - rapid freeze methods were used to trap short-lived intermediates in isopentane at -110 °C. By freeze-drying the
flash-frozen reaction mixture, high quality Raman difference spectra of the freeze-dried material were obtained in the presence and absence of substrate. These provided the Raman spectra of the substrate-based intermediates undergoing catalysis. With a commercial rapid mix apparatus (KinTek RQF-3), we are able to trap intermediate at 25 milliseconds and longer after mixing using approximately 0.5 milligram of protein at each time point.

β-lactamases confer antibiotic resistance to bacteria by hydrolyzing antibiotic drugs such as penicillins and cephalosporins (81, 82). To date, more than 1600 unique β-lactamases have been reported (17). According to their amino-acid sequences (Ambler method), β-lactamase enzymes are generally divided into four classes, classes A to D (83, 84). Among the well-studied class A enzymes, SHV-1 and TEM-1 are the most commonly encountered enzymes in Gram-negative bacteria. To combat the hydrolytic activity of β-lactamase, clinical inhibitors of β-lactamase have been developed, such as clavulanic acid, sulbactam and tazobactam. Unlike β-lactam antibiotics, these competitive inhibitors can inhibit the enzyme by forming long-lived or irreversible intermediates in the active site of the enzyme (61). Unfortunately, bacteria also acquire resistance to these inhibitors due to mutations in β-lactamases.

For a decade, our laboratory has developed Raman crystallography to track reactions of the clinical inhibitors in single crystal of β-lactamase in real time (61). The spectroscopic analysis provides information about the chemical identity of the intermediates, their relative populations, and their rates of formation and disappearance. However, time-resolution in crystal studies is presently >1 min. Although inhibitor design will usually depend on the analysis of long-lived complexes in the crystal, it is
imperative to understand early events on the reaction pathway in solution because these can reveal the identities of “stable” complexes.

II.2 MATERIALS AND METHODS

*Raman spectroscopy* - SHV-1 β-lactamase was purified as previously described (61, 85, 86). The Raman spectrum of a SHV-1 single crystal with tazobactam was obtained as described before (53, 61).

For the rapid mix - rapid freeze experiment, we used a slightly modified KinTek instrument model RQF-3. Reactions were initiated by mixing SHV-1 enzyme (5 mg/ml in 2 mM HEPES, pH 7) with tazobactam in a 1:4 molar ratio. The turnover number is 4 - 5 and using a 4 fold excess of tazobactam ensures maximum active-site occupancy. The mixture then traversed through different reaction loops with pre-determined time delay, and the reaction mixture was rapidly frozen by injecting into isopentane at about -110 °C, within a tube that was immersed in liquid nitrogen. Isopentane was removed from the ice, and by freeze-drying the frozen material, the lyophilized powder was obtained that constituted Raman samples. The powder was examined using Raman microscope (87) (80 mW of 647.1 nm was used as the excitation source and each data set was acquired using 1 × 100 seconds exposure). Based on the reaction loops and exit line volume (that determine the distance of travel for the mixture of enzyme and substrate prior to freezing) and the rpm of the drive motor, we calculated the time of the reaction. The reaction mixture travels 1 to 2 cm in the air after the exit loop to enter the cold isopentane. This gap can produce the error in the time points between 1 to 3 milliseconds based on the rpm of the drive motor.
Absorbance studies- Tazobactam was bought from Sigma Company. A stock solution of the inhibitor at 20 mM in 10 mM HEPES buffer (pH 7.5) was prepared for UV-absorbance studies. Potency of SHV-1 was verified using the colorimetric β-lactamase substrate nitrocefin (Becton Dickson). Nitrocefin gives rise to an intense peak at 482 nm, \( \Delta \varepsilon_{482} = 17400 \, \text{M}^{-1}\text{cm}^{-1} \) after it is hydrolyzed. The final concentrations of the SHV-1 protein (before and after freeze-drying) were both 1 µM. The final concentration of tazobactam in the reaction system was 1 µM (1 : 1 ratio) or 10 µM (1 : 10 ratio). After SHV-1 (untreated or freeze-dried), tazobactam and nitrocefin were rapidly mixed, the hydrolytic rate was measured by the change of the absorbance at 482 nm.

**Scheme II. 1** Partial reaction scheme for tazobactam and class A beta lactamase enzymes.
II.3 RESULTS AND DISCUSSION

II.3.1 Tazobactam reactions with SHV-1 in single crystals

We illustrate the adaptation of the rapid mix - rapid freeze method using the reaction between tazobactam, a commercially available "suicide inhibitor" of β-lactamases, and the SHV-1 β-lactamase, an important resistance determinant in *Klebsiella pneumoniae*. We have extensive experience on this reaction in single crystals (61, 64, 65, 88-91) and here we compare the reaction intermediates formed in crystal and solution environments. A partial reaction scheme is shown in Scheme II.1.

When following the reaction in a single crystal of wild-type SHV-1, we showed that there is an equilibrium between major populations of protonated imine (Scheme II.1), *cis* and *trans*-enamine (Scheme II.1) (61, 64). The Raman difference spectrum for tazobactam reacting in a single crystal of SHV-1 β-lactamase is shown in Figure II.1. The crucial underpinnings of the band assignments came from combined Raman and X-ray crystallographic experiments. Based on the literature and kinetic data, the peaks near 1595 cm\(^{-1}\) reported in the first paper on tazobactam, sulbactam and clavulanic acid reacting in crystals of the E166A variant of SHV-1 were assigned to the stretching vibration of the –O–C(=O)–C=CH– fragment of *trans*-enamine (53). This prediction and the presence of the *trans*-enamine species was confirmed by subsequent X-ray analysis (92, 93). Detailed analysis from Gaussian calculations (Table II.1, below) has confirmed that the 1589 cm\(^{-1}\) feature is a characteristic marker band of the *trans*-enamine, and that the 1658 cm\(^{-1}\) band is due to the pro-*trans* imine (Scheme II.1) (61).
Figure II. 1 Partial Raman difference spectrum of the intermediate from the tazobactam reaction with SHV-1, in a single crystal after 20 minutes of soaking in 20 mM tazobactam.

In Figure II.1, the narrow bands at 1658 and 1589 cm\(^{-1}\) (that have 17 and 20 cm\(^{-1}\) widths at half height, respectively) suggest that the \textit{trans}-enamine is in a single, well-defined conformation. The weak feature at 1678 cm\(^{-1}\) may be due to a minor population of pro-\textit{cis} imine. In Kalp \textit{et al.} (61), it was assigned to an amide I feature; however, the present band is too narrow to unambiguously make this assignment. Similarly, it is possible that the profile at 1589 cm\(^{-1}\) also contains a contribution from \textit{cis}-enamine.

The tazobactam triazolyl ring has a fairly intense mode near 1290 cm\(^{-1}\) in aqueous solution (Figure II.3, upper trace). In Figure II.1, this mode appears to have multiple components and we ascribe these to several slightly different local binding sites on the
enzyme for the triazole ring each giving rise to slightly different ring frequencies. Triazole also has a weak mode near 1240 cm\(^{-1}\). The feature in Figure II.1 at 1241 cm\(^{-1}\) likely contains a contribution from the triazole mode and also from a NH bending mode from the enamine and / or protein peptide bonds.

**II.3.2 Freeze-drying does not affect protein secondary structure and catalytic property.**

Before comparing the results between *in crystallo* and in rapid mixing, we first investigated the effect of freeze-drying on the enzyme. We compared the Raman spectra of the SHV-1 crystal and freeze-dried powder. The resulting spectra are shown in Figure II.2 and are very similar. The similarities in the amide I (1660 cm\(^{-1}\) region), amide III (1220 - 1320 cm\(^{-1}\) region) profiles indicate that there are no major changes in secondary structure after freeze-drying. This conclusion is supported by the similar profiles *circa* 530 cm\(^{-1}\). The latter is due to the S-S mode involving cysteine residues at positions 77 and 123 (85), and the S-S stretch mode is sensitive to changes in conformation about these linkages (94). The small differences in Figure II.2 are probably due to minor changes in loops and disordered sequences occurring upon dehydration.
Figure II. 2 Comparison of Raman spectrum of SHV-1 enzyme in crystal (upper trace) and freeze-dried powder (lower trace).

To ensure that freeze-drying did not affect the function of SHV-1, the hydrolysis of nitrocefin by wild-type SHV-1 before and after freeze-drying was measured. Nitrocefin is used as a chromogenic substrate for SHV-1, which gives rise to an intense peak at 482 nm after it is hydrolyzed. Two identical aliquots of SHV-1 enzyme were used; one was tested immediately and the other one was lyophilized at -48 °C. The lyophilized enzyme was re-dissolved in water and the concentration was adjusted to equal that of the non-lyophilized sample. The non-lyophilized SHV-1 enzyme (1 µM) was assayed with nitrocefin in the absence or the presence of 1 µM or 10 µM of tazobactam. The change of the intensity at 482 nm was recorded to reflect the hydrolytic activity of SHV-1 enzyme.
on nitrocefin. The results are compared in Figure I.12 in Chapter I to those using freeze-dried SHV-1 that was treated in exactly the same manner. The three pairs of kinetic traces overlap precisely, indicating SHV-1 retains essentially 100% activity in the freeze-drying process.

II.3.3 *Tazobactam reactions with SHV-1 in solution forms stable trans-enamine.*

Figure II.3 compares the Raman difference spectra for flash-frozen, acyl-enzyme intermediates, between 25 and 1000 milliseconds after mixing, with the spectrum of free aqueous tazobactam (upper trace). For the intermediates the broad band near 1595 cm<sup>-1</sup> (approximately 45 cm<sup>-1</sup> width at half height) is compelling evidence for the presence of enamine species, and the large bandwidth suggests a mixture of *cis* and *trans* species (Table II.1). In Kalp *et al.* (*61*), similar sulbactam intermediates were suggested to have enamine marker bands at 1588 and 1605 cm<sup>-1</sup>, respectively. The sharp triazole peak at 1287 cm<sup>-1</sup> (19 cm<sup>-1</sup> width at half height) indicates that "natural line width" (of sharp features) in the lyophilized powder is approximately 20 cm<sup>-1</sup> and is evidence that the broad line width of 1596 cm<sup>-1</sup> in Figure II.3 may be due to multiple isomers. The protonated imine peaks at 1658 (pro-*trans*) and 1673 cm<sup>-1</sup> (pro-*cis*) in Figure II.3 are narrow and if assigned correctly suggest a higher relative population of the pro-*trans* imine isomer in solution compared to pro-*cis*.

The relative population of pro-*trans* imine in the crystal (Figure II.1) appears to be higher compared to the population of pro-*trans* imine in solution (Figure II.3), and *in crystallo*, the intense imine peak remains at 60 min. Since in solution, the imine feature almost disappears at one second, the "lifetime" of the imine in the crystal is >10<sup>3</sup> times
longer than in solution. Thus, in solution, at one second after mixing, the imine isomers seem to have essentially converted to enamine. We have detected the formation of enamine isomers quickly, within 25 milliseconds after mixing, which remain essentially unchanged after one second. These isomers are accompanied by the expected imine predecessors that convert to enamine within one second.
Figure II. 3 Partial Raman spectrum of tazobactam (20 mM, pH 7.0, top) and Raman difference spectra of tazobactam reacting with SHV-1; trapped intermediates in lyophilized powder between 25 and 1000 milliseconds after mixing. In the tazobactam spectrum the 1780 cm\(^{-1}\) feature corresponds to the stretch of carbonyl group in \(\beta\)-lactam ring.
**Table II.** Raman peak assignments for the major peaks in tazobactam/SHV-1 difference spectra shown in Figure II.3.

<table>
<thead>
<tr>
<th>Species</th>
<th>Wavenumber (cm(^{-1}))</th>
<th>Peak assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>unreacted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tazobactam</td>
<td>1780</td>
<td>C=O of β-lactam ring</td>
</tr>
<tr>
<td></td>
<td>1491</td>
<td>C=C of triazolyl ring</td>
</tr>
<tr>
<td></td>
<td>1401</td>
<td>CO(_2)(^{-}) (symmetric stretch) at C3 position</td>
</tr>
<tr>
<td></td>
<td>1292</td>
<td>breathing of triazolyl ring</td>
</tr>
<tr>
<td></td>
<td>1233</td>
<td>N=N of triazolyl ring</td>
</tr>
<tr>
<td>imine</td>
<td>1658</td>
<td>C=NH(^{+}) of imine</td>
</tr>
<tr>
<td>trans-enamine</td>
<td>1596</td>
<td>–O–C(=O) –C=–NH– stretch of trans-enamine</td>
</tr>
</tbody>
</table>

In order to measure the turnover number for SHV-1 β-lactamase enzyme, we mixed SHV-1 and tazobactam in different molar ratios (from 1 : 1 to 1 : 10). The reaction mixture was frozen after 20 seconds of mixing by injecting into isopentane at -110 °C. The Raman difference spectrum of each sample was acquired after freeze-drying of the flash-frozen materials. The intensities of the peaks around 1595 cm\(^{-1}\) in the difference spectra (enamine species) were measured, and the 1450 cm\(^{-1}\) protein peak in original spectra (before subtraction) was used as an internal standard. The intensity ratio was plotted against the ratio of tazobactam to enzyme, and the plot is shown in Figure II.4. The enamine peak intensity is constant after reaching the ratio of 1:4 to 1:5, and it shows
the turnover number is between 4 and 5, which agrees with the value of 5 obtained from kinetic methods (95).

Figure II. 4 Direct measurement of turnover number for tazobactam reaction with SHV-1 enzyme. It demonstrates that the enzyme is completely inhibited giving rise a maximum enamine population after 4 - 5 equivalents of tazobactam have been hydrolyzed.

II.4 CONCLUSIONS

In summary, we have reported the combination of rapid mixing - rapid freezing, and freeze-drying, with Raman microscopy to investigate the enzyme-substrate reaction. The sensitivity of Raman signal is enhanced by freeze-drying the sample. The results due to the binding of inhibitor to the active site of enzyme in rapid mixing show analogous intermediates compared to those in crystals. However, the lifetimes, in relative and absolute terms, are very different. The present results confirm the presence of a stable trans-enamine in solution and this is the species that can be used for strategy-based drug
design (55). The method outlined herein should be widely applicable to other enzyme-substrate reaction.
Chapter III: Detecting a Quasi-stable Imine Species on the Reaction Pathway of SHV-1 β-Lactamase and 6β-(Hydroxymethyl)penicillanic Acid Sulfone

**ABSTRACT:** For the class A β-lactamase SHV-1, the kinetic and mechanistic properties of the clinically used inhibitor sulbactam are compared with the sulbactam analog substituted in its 6β position by a -CH₂OH group (6β-(hydroxymethyl)penicillanic acid). The 6β substitution improves both *in vitro* and microbiological inhibitory properties of sulbactam. Base hydrolysis of both compounds was studied by Raman and NMR spectroscopies and showed that lactam ring opening is followed by fragmentation of the dioxothiazolidine ring leading to formation of the iminium ion within 3 minutes. The iminium ion slowly loses a proton and converts to *cis*-enamine (which is a β-aminoacrylate), in 1 hour for sulbactam, and in 4 hours for 6β-(hydroxymethyl) sulbactam. Rapid mix - rapid freeze Raman spectroscopy was used to follow the reactions between the two sulfones and SHV-1. Within 23 milliseconds a 10-fold excess of sulbactam was entirely hydrolyzed to give a *cis*-enamine product. In contrast, the 6β-(hydroxymethyl) sulbactam formed longer-lived acyl-enzyme intermediates that are a mixture of imine and enamines. Single crystal Raman studies, soaking in and washing out unreacted substrates, revealed stable populations of imine and *trans*-enamine acyl enzymes. The corresponding X-ray crystallographic data are consonant with the Raman data and also reveal the role played by the 6β-hydroxymethyl group in retarding hydrolysis of the acyl enzymes. The 6β-hydroxymethyl group sterically hinders approach of the water molecule as well as restraining the side chain of E166 that facilitates hydrolysis.

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III.1 INTRODUCTION

The production of β-lactamases is the major cause of failure of β-lactam-based therapy against Gram-negative bacteria. Since the first β-lactamase was identified in Escherichia coli >70 years ago (96), more than 1600 different enzymes have been reported. Currently, β-lactamases are grouped into four classes (A through D) based on the Ambler classification (18). New β-lactamases generated in the Enterobacteriaceae family, the Pseudomonas, Acinetobacter, and Klebsiella genera, stimulated the development of novel β-lactam antibiotics from penicillins to extended-spectrum cephalosporins (e.g. ceftazidime, ceftriaxone and cefepime) and to carbapenems (imipenem, meropenem, ertapenem, and doripenem). Regrettably, an emerging number of community and hospital-acquired E. coli and K. pneumoniae have already shown resistance to this last-line of defense (cephalosporins and carbapenems) (19). An urgent need exists to design more effective therapies.

In most clinical applications, mechanism-based inhibitors (clavulanate, sulbactam, or tazobactam) against β-lactamases are applied in combination with a partner β-lactam antibiotic. The inhibition mechanism of these inhibitors has been widely studied (61). When the inhibitors react with the target β-lactamase, the carbonyl carbon of the β-lactam ring is attacked by a Ser-OH forming an acyl-enzyme followed by the opening of the adjacent dioxothiazolidine ring. Study of the reaction pathways postulates that an iminium ion (often referred to as an imine) intermediate is then rapidly formed that tautomerizes to an enamine. The latter often forms a stable acyl-enzyme complex and inhibits the enzyme. The structures of acyl-enzyme complexes have been solved showing that a trans-enamine is covalently bound to the active site (92, 93). This finding led to
new inhibitor design aimed at producing a more stable trans-enamine in the active site. SA2-13, a penam sulfone derivative, is one such structure-based inhibitor that can stabilize the trans-enamine intermediate to inhibit class A β-lactamases (55). However, commercial inhibitors narrowly target the class A enzymes. New clinically useful β-lactamase inhibitors need to have a significantly broader inhibitory spectrum, ideally targeting all serine β-lactamas (classes A, C and D). Recent studies with avibactam, a diazabicyclooctane, DBO, inhibitor of the non β-lactam class, support the importance of this strategy.

In 1999, Bitha et al. showed the addition of a 6-hydroxymethyl group in sulbactam improves the activity against both TEM-1 (175-fold increase) and AmpC (57-fold increase) β-lactamas (97, 98). This 6β-hydroxymethyl sulbactam (PSR-3-283A) also restores the activity of piperacillin in vitro (MIC) and in vivo (ED50) against both class A and C β-lactamase producing microorganisms. Bitha et al. proposed that the approach of the hydrolytic water may be affected by the presence of 6β-hydroxymethyl group. In 2012, Papp-Wallace et al. (99) investigated the reaction pathway of PSR-3-283A with TEM-1 and PDC-3, the AmpC of the pathogen Pseudomonas aeruginosa using mass-spectrometry. Their results indicate that the improved efficiency of PSR-3-283A may be due to loss of the hydrolytic water after acylation. However, the underlying mechanism was not studied in detail, partly because the reaction occurs very quickly.

Recently, our laboratory developed a new protocol to study this rapid reaction. This approach combines rapid mix – rapid freeze with Raman spectroscopy enabling us to capture the intermediates or products on the millisecond time frame (54). The rapid reaction - Raman approach has the advantage that it provides time resolved structural
data. Herein, using this new protocol we tested PSR-3-283A with the clinically important β-lactamase, SHV-1, one of the most common class A β-lactamases detected in clinical isolates of *E. coli* and *K. pneumoniae*. By comparing PSR-3-283A with sulbactam, we show that a large population of imine species is formed as a relatively stable intermediate in the reaction between PSR-3-283A and SHV-1, but only hydrolyzed cis-enamine product is detected with sulbactam. This is the first time we have captured a quasi-stable imine in the active site because with the commercial inhibitors, the imine intermediate is usually very short-lived. A 1.37 Å crystal structure of SHV-1 in complex with PSR-3-283A shows the 6-hydroxymethyl group in the imine and enamine complex forms extra H-bonds with the deacylation water molecule and the general base E166 and therefore hampers deacylation. The different properties between sulbactam and PSR-3-283A due to the additional 6β-hydroxymethyl group provide an approach to improving or designing more versatile β-lactamase inhibitors.

**III.2 MATERIALS AND METHODS**

*Antibiotics and β-lactamase inhibitors.* The antibiotics used in this study were obtained from their respective manufacturers as indicated: ampicillin, cephalothin, and clavulanic acid, potassium salt, from Sigma (St. Louis, MO); sulbactam, sodium salt, from Pfizer (Groton, CT); tazobactam, sodium salt, from Chem-IMPEX (Wood Dale, IL); and nitrocefin from Becton Dickinson (Franklin Lakes, NJ). The sodium salt of PSR-3-283A (263 Da) and 6-D,D-sulbactam were synthesized in the laboratory of John D. Buynak. Chemical structures of β-lactamase inhibitors are represented in Figure III.1.
Figure III. 1 Chemical structures of the clinical inhibitors, tazobactam, sulbactam and clavulanic acid, and PSR-3-283A.

Subcloning, expression, and purification- The subcloning, expression, and purification of wt SHV-1 was carried out similarly to that previously described (86, 100, 101). Briefly, the bla<sub>SHV-1</sub> gene was subcloned into the phagemid vector pBC SK (-) (Stratagene) with a periplasmic leader sequence. This construct was transformed into <i>Escherichia coli</i> DH10B cells (Stratagene). Cells were grown overnight in lysogeny broth (LB) supplemented with 20 μg/ml chloramphenicol. Cells were lysed using a periplasmic lysis protocol adapted from Peripreps Periplasting Kit (EpiCentre). Briefly, cell pellets are resuspended in a buffer containing 200 mM Tris-HCl pH 7.5, 20% sucrose, 1 mM EDTA and 4 mg/g dry cell weight lysozyme. After a 5 minute incubation without stirring, the reaction is diluted 1.5 fold with water and left on ice for seven minutes. The lysate is cleared by centrifugation (12,000 g) at 4 °C for 15 minutes. The lysate was subsequently purified using preparative isoelectric focusing (pIEF) gel chromatography. β-Lactamase
was detected using nitrocefin (Calbiochem), a chromogenic \( \beta \)-lactamase substrate. Nitrocefin "positive" pIEF fractions were applied to Q-Sepharose anion exchange column using either 20 mM HEPES pH 7.0 or 20 mM BisTris pH 6.5 as the equilibration buffer with 1 M NaCl or 1 M KCl added as the elution buffer. In both the HEPES/NaCl and BisTris/KCl conditions the \( \beta \)-lactamase eluted in the flow-through while the majority of impurities bound the column. \( \beta \)-lactamase positive fractions were applied to a Superdex75 gel filtration column as the final purification step. Protein purity was assessed by SDS-PAGE and pure protein was concentrated to 5 mg/ml.

**Kinetics**- Steady state kinetics were performed on an Agilent 8453 diode array spectrophotometer (Palo Alto, CA) in 10 mM phosphate-buffered saline (pH 7.4). \( V_{\text{max}} \) and \( K_m \) were determined from initial steady-state velocities for nitrocefin (NCF), \( \Delta \varepsilon_{482} = 17,400 \text{ M}^{-1}\text{cm}^{-1} \). The kinetic parameters were obtained using iterative non-linear least-squares fit of the data to the Henri-Michaelis-Menten equation using Origin 8.0 (OriginLab, Northampton, MA) according to Equation 1:

\[
v = \frac{V_{\text{max}} [S]}{(K_m + [S])}
\]  

\[ (1) \]

\( IC_{50} \), defined as the inhibitor concentration resulting in a reduction of NCF (100 \( \mu \text{M} \)) hydrolysis by 50\%, was determined by measurements of initial velocities after 5 minutes preincubation of enzyme with inhibitor (66). The first-order rate constant for enzyme inactivation, \( k_{\text{inact}} \), was determined by monitoring the reaction time courses in the presence of increasing concentrations of inactivators. A fixed concentration of enzyme, nitrocefin, and increasing concentrations of inactivator were used in each assay. The \( k_{\text{obs}} \) for inactivation was determined graphically as the reciprocal of the ordinate of the intersection of the straight lines obtained from the initial, \( v_0 \), and final, \( v_f \), steady-state
velocities. Each $k_{obs}$ was plotted versus inhibitor concentration, $I$, and fit to Equation 2 to determine $k_{inact}$ and $k_{inact}/K_I$ (the second order rate constant for reaction of free enzyme with free inhibitor to give inactive enzyme):

$$k_{obs} = k_{inact} [I]/(K_I + [I]) \quad (2)$$

Partition ratios ($k_{cat}/k_{inact}$) were determined as the ratio of inhibitor concentration to enzyme concentration that was necessary to decrease the enzyme activity by 95%. Partition ratios were determined after a 24 hr incubation with increasing concentrations of the inhibitor. Incubations were done in a final volume of 300 µl and 25 µl of this reaction mixture were added to a 1 ml final volume to determine the residual enzyme activity using 100 µM nitrocefin.

**Susceptibility testing.** For disc diffusion testing, *E. coli* DH10B pBCSK(-) with and without $bla_{SHV-1}$ were used. Disc diffusion assays were conducted using the guidelines of Clinical and Laboratory Standards Institute (CLSI) (102). Solutions of cephalothin (30 µg) and inhibitor (30 µg) were pipetted onto discs based on the combinations shown in Table III.2 and allowed to dry for 1 h. Colonies were directly re-suspended into sterile water equivalent to a 0.5 McFarland standard, and used to inoculate Mueller Hinton (MH) agar plates. The discs were carefully placed on each plate. The bacteria were grown at 37 °C for 18 h and zone diameters were measured.

**Raman studies in solution.** For the rapid mix - rapid freeze experiment, we used a slightly modified KinTek instrument model RQF-3. Reactions were initiated by mixing SHV-1 enzyme (5 mg/ml in 2 mM HEPES, pH 7) with β-lactamase inhibitors (sulbactam, 6-D,D-sulbactam, PSR-3-283A) in a 1:10 molar ratio. The mixture then traversed through different reaction loops with predetermined time delay, and the reaction mixture was
rapidly frozen by injection into isopentane at about -110 °C, within a tube that was immersed in liquid nitrogen. Isopentane was removed from the ice, and by freeze-drying the frozen material, the lyophilized powder was obtained that constituted Raman samples. The powder was examined using the Raman microscope (87) (80 mW of 647.1 nm was used as the excitation source and each data set was acquired using 1 × 100 seconds exposure). On the basis of the reaction loops and exit line volume (that determine the distance of travel for the mixture of enzyme and substrate prior to freezing) and the rpm of the drive motor, we calculated the time of the reaction. The reaction mixture travels 1 to 2 cm in the air after the exit loop to enter the cold isopentane. This gap can produce the error in the time points between 1 to 3 milliseconds based on the rpm of the drive motor. A schematic picture of rapid mix - rapid freeze has been described previously (54).

**NMR spectroscopy.** ¹H NMR spectra were acquired on a Varian VXR Inova 600 MHz spectrometer. The experiments were run at 20 °C in the solvent of D₂O. After sulbactam was incubated with 1 M NaOH for different time period, approximately 1 ml of the solution was transferred to a NMR tube. The sample tube was then inserted in the magnet. The experiment used a standard 2 pulses and a 30 degrees pulse was used. The delay before the application of the pulse was 1 s and the acquisition time of the pulse was 3.5 s. There were 64 repetitions and the total time was 4 min and 49 s.

**Crystallization and soaking**- Crystals of wt SHV-1 β-lactamase were grown using hanging drop vapor diffusion over a well solution containing 21-30% PEG6000 and 0.1 M HEPES pH 6.8-8.2. The protein solution (5 mg/ml) was combined with the Cymal-6 additive (Hampton, final concentration 0.56 mM). 5 μl drops were set using a 1:1 protein:well solution ratio with crystals growing in 1-3 weeks. Crystals were soaked in a drop
containing mother liquor and 50 mM PSR-3-283A for 15 minutes. After soaking, crystals were transferred to a cryoprotectant solution containing mother liquor and inhibitor supplemented with 25% methyl-2,4-pentanediol (MPD) before being flash frozen in liquid nitrogen.

*Data collection and refinement*- All data was collected on BL9-2 at the Stanford Synchrotron Radiation Lightsource (SSRL), Stanford University, Menlo Park, CA. Data were integrated and scaled using HKL2000 *(103)*. Structures were determined by isomorphous replacement using the wt SHV-1 apo structure (PDB ID: 1SHV). Restrained refinement was carried out using REFMAC5 *(104)* and COOT *(105)* was used for model building and manual refinement. Cymal-6 detergent molecules were included in the refinement. After refinement of side chains and addition of solvent molecules, difference electron density was present for part of the PSR-3-283A inhibitor bound in the active site. Inhibitor coordinate and topology files were generated using PRODRG server *(106)*. The inhibitor was modeled in a linearized, extended conformation with the tail atoms being less ordered. The more electron-dense sulfone moiety of the inhibitor was modeled into the strongest difference density (6.5σ) in this region. Data collection and refinement statistics are found in Table III.1. Coordinates and structure factors for the enzyme inhibitor complexes have been deposited into the Protein Data Bank (PDB ID: 4R3B). RMSDs were calculated using the LSQKAB utility in CCP4 *(107)*.
Table III. 1 X-ray data collection and refinement statistics.

<table>
<thead>
<tr>
<th>Data Collection and Refinement Statistics for SHV-1 PSR-3-283A complex</th>
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</thead>
<tbody>
<tr>
<td><strong>Data Collection Statistics</strong></td>
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<tr>
<td>Space Group</td>
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<td>Cell Dimensions</td>
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<tr>
<td>a, b, c (Å)</td>
</tr>
<tr>
<td>α, β, γ (Å)</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
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<tr>
<td>Resolution (Å)²</td>
</tr>
<tr>
<td>R_{sym}</td>
</tr>
<tr>
<td>I/σI</td>
</tr>
<tr>
<td>Completeness (%)</td>
</tr>
<tr>
<td>Redundancy</td>
</tr>
<tr>
<td><strong>Refinement Statistics</strong></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
</tr>
<tr>
<td>No. of Reflections</td>
</tr>
<tr>
<td>R_{work}/R_{free}</td>
</tr>
<tr>
<td>No. of atoms</td>
</tr>
<tr>
<td>(protein/ligand/water)</td>
</tr>
<tr>
<td>RMSD</td>
</tr>
<tr>
<td>Bond length (Å)</td>
</tr>
<tr>
<td>Bond angle (°)</td>
</tr>
<tr>
<td>Average B-factors (Å²)</td>
</tr>
<tr>
<td>protein</td>
</tr>
<tr>
<td>Ligands</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>Ramachandran plot statistics (%)</td>
</tr>
<tr>
<td>Core</td>
</tr>
<tr>
<td>Allowed regions</td>
</tr>
<tr>
<td>Gen. Allowed regions</td>
</tr>
<tr>
<td>Outliers</td>
</tr>
</tbody>
</table>
III.3 RESULTS

III.3.1 Kinetic parameters

The kinetic parameters for PSR-3-283A with the target enzyme SHV-1 β-lactamase, were measured and the results are compared in Table III.2 with those for the clinically used inhibitors tazobactam, sulbactam and clavulanate.

The observed IC$_{50}$ (0.32 ± 0.03 μM) of PSR-3-283A is 10-fold and 3-fold higher than those of tazobactam (0.030 ± 0.003 μM) and clavulanate (0.10 ± 0.02 μM), respectively. However, it is 10-fold lower than that of sulbactam (3.4 ± 0.5 μM).

Additionally, the $K_I$ (0.56 ± 0.05 μM) is about 3-fold higher than that of tazobactam (0.16 ± 0.03 μM), but 3-fold lower than those of clavulanate (2.0 ± 0.2 μM) and sulbactam (1.5 ± 0.3 μM), respectively. This indicates that PSR-3-283A has a higher affinity compared with clavulanate or sulbactam for SHV-1. The $k_{inact}/K_I$ ratio for PSR-3-283A is comparable to that of clavulanate and 4-fold higher than that of sulbactam. The partition ratio at 24h for PSR-3-283A ($t_n = 80$) is comparable to those of tazobactam ($t_n = 60$) and clavulanate ($t_n = 40$) and at least 100-fold lower than that of sulbactam ($t_n = 10,000$). The kinetic data with SHV-1 β-lactamase show that PSR-3-283A has not only increased affinity but also greater inactivation efficiency compared with sulbactam. This is likely attributable to the additional 6-hydroxymethyl group in PSR-3-283A and its ability to resist catalysis.
Table III. 2 Kinetic parameters of SHV-1 β-lactamase with various inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>$IC_{50}$ (5min) (µM)</th>
<th>$k_{inact}$ (s$^{-1}$)</th>
<th>$K_I$ (µM)</th>
<th>$k_{inact}/K_I$ (µM$^{-1}$s$^{-1}$)</th>
<th>$K_{cat}/k_{inact}$ (24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clavulanic acid</td>
<td>0.10 ± 0.02</td>
<td>0.034 ± 0.001</td>
<td>2.0 ± 0.2</td>
<td>0.017 ± 0.002</td>
<td>40</td>
</tr>
<tr>
<td>Tazobactam</td>
<td>0.030 ± 0.003</td>
<td>0.10 ± 0.01</td>
<td>0.22 ± 0.03</td>
<td>0.45 ± 0.07</td>
<td>60*</td>
</tr>
<tr>
<td>PSR-3-283A</td>
<td>0.32 ± 0.03</td>
<td>0.010 ± 0.001</td>
<td>0.56 ± 0.05</td>
<td>0.018 ± 0.002</td>
<td>80</td>
</tr>
<tr>
<td>Sulbactam</td>
<td>3.4 ± 0.5</td>
<td>0.006 ± 0.001</td>
<td>1.5 ± 0.3</td>
<td>0.004 ± 0.001</td>
<td>10,000</td>
</tr>
</tbody>
</table>

* Previously reported as 5. Difference likely due to potency of the product.

III.3.2 Susceptibility testing

In order to test PSR-3-283A's ability to synergistically help antibiotics kill bacterial cells, disc diffusion assays were conducted and zone diameters were measured for *E. coli* DH10B without or with pBCSK(-) *bla*$_{SHV-1}$ (Table III.3). The *E. coli* strain expressing SHV-1 was resistant to the antibiotic cephalothin with a zone size of 6 mm. When cephalothin was combined with clinical inhibitors or PSR-3-283A, the latter displayed slightly better synergy (24 mm) than tazobactam (16 mm) and much better than sulbactam (10 mm).
Table III. 3 Disc diffusion assays.

<table>
<thead>
<tr>
<th></th>
<th>E. coli DH10B pBCSK control (mm)</th>
<th>E. coli DH10B pBCSK SHV-1 (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalothin(^a)</td>
<td>34</td>
<td>6</td>
</tr>
<tr>
<td>Cephalothin-Sulbactam(^a)</td>
<td>34</td>
<td>10</td>
</tr>
<tr>
<td>Cephalothin-Clavulanic acid(^a)</td>
<td>34</td>
<td>36</td>
</tr>
<tr>
<td>Cephalothin-Tazobactam(^a)</td>
<td>36</td>
<td>16</td>
</tr>
<tr>
<td>Cephalothin-PSR-3-283A(^a)</td>
<td>40</td>
<td>24</td>
</tr>
<tr>
<td>PSR-3-283A alone</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) 30 µg of cephalothin and 30 µg of each inhibitor were used.

III.3.3 Base hydrolysis

Before investigating the enzyme catalyzed reactions of PSR-3-283A and sulbactam, we followed base hydrolysis of the two inhibitors by normal Raman spectroscopy to aid us in understanding the mechanism of inactivation of β-lactamases. The involvement of a serine hydroxy function in the enzyme reaction has prompted researchers to employ parallel base hydrolysis to study rearrangement of β-lactamase inhibitors (108). Herein, the results can be understood in terms of Scheme III.1. The major Raman peak assignments follow those we have derived in order to characterize imine and enamine species in the active site of SHV-1 β-lactamase (61). The key assignments are listed in Table III.4.
Previous work in our laboratory by Kalp et al. (61) studied the detailed mechanisms when sulbactam, clauvalanate or tazobactam react with SHV-1 within a crystal. Much effort has been expended to assign the Raman peaks of imine, cis- and trans-enamine species in the reaction pathway. Using a biochemical reagent or isotopic substitutions, Kalp et al. provide solid evidence showing that protonated imine (C=NH\textsuperscript{+}) has characteristic peak near 1656 cm\textsuperscript{-1}, trans-enamine (C=C) near 1595-1615 cm\textsuperscript{-1} and cis-enamine (C=C) near 1580-1595 cm\textsuperscript{-1} where the assignments are stretching modes with a large contribution from the double bond. These assignments are also strongly supported by Kalp’s, and our, recent quantum mechanical calculations using the Gaussian program. Taken together these constitute the foundation for our assignments in the base hydrolysis studies and reactions of PSR-3-283A and SHV-1.

In the base hydrolysis experiment, 2 µL 20 mM sulbactam were mixed with 2 µL 1M NaOH and the Raman spectra were then recorded in real time. In Figure III.2A, the reaction appears to display a strict two-step mechanism. After sulbactam and NaOH were mixed, the OH\textsuperscript{-} attacks the carbonyl group in the β-lactam ring and the imine rapidly accumulates and reaches a maximum at less than 3 min, as evidenced by the intense peak at 1630 cm\textsuperscript{-1}. The 1630 cm\textsuperscript{-1} peak, based on Gaussian calculations, is assigned to C=N in the deprotonated imine (Scheme III.1, Table III.4) Opening of the five-membered ring is supported by the absence of the 632 cm\textsuperscript{-1} peak (C\textsubscript{5}-S\textsubscript{1} stretch). The appearance of the
profile at 598/535 cm\(^{-1}\) originates from the deprotonated -SO\(_2\)\(^-\)\((109, 110)\) in the hydrolyzed imine and \textit{cis}-enamine species (3 and 4, Scheme III.1). The accumulation of imine at 3 min also means that the rate-limiting step is the transition from imine to enamine. From 3 to 60 min, the peak 1630 cm\(^{-1}\) decreases while the 1581 cm\(^{-1}\) feature continues to increase, indicating more hydrolyzed \textit{cis}-enamine is formed (Table III.4). At 60 min, all imine molecules have been converted to the \textit{cis}-enamine. This two-step reaction is also supported by the NMR analysis (Figure III.S1). In "supplemental material", for base hydrolysis we assign resonances in the \(^1\)H NMR to imine and \textit{cis}-enamine isomers. They interconvert with the same time dependence that we have characterized in the base hydrolysis measured by Raman analysis.

In the hydrolysis of PSR-3-283A by 1 M NaOH (Figure III.2B), the reaction displays a similar two-step mechanism but the imine-enamine tautomerization rate is eight times slower than in sulbactam. At 3 min, all PSR-3-283A molecules are hydrolyzed to imine species (1632 cm\(^{-1}\)) and the latter still dominates at 1 hour. After 4 hours, the ratio of imine : enamine (1631 : 1595 cm\(^{-1}\)) is \(\sim 1:1\) (data not shown). Thus, for PSR-3-283A undergoing base hydrolysis, the imine species requires a higher energy barrier for conversion to enamine compared to that for the sulbactam derived imine and enamine. There are two possible explanations for this slow conversion. One is that the bulky –CH\(_2\)OH replaces one of the two hydrogen atoms, thus essentially reducing the rate by half; the other is that the –CH\(_2\)OH group interferes with the approach of a OH\(^-\) base to the remaining hydrogen at C6 position, slowing the overall process. The imine is important because in the following sections we will show how its stability is increased further in SHV-1 \(\beta\)-lactamase's active site by interactions with the 6-hydroxymethyl.
Table III. 4 Peak assignment derived from Gaussian calculations.

<table>
<thead>
<tr>
<th>Species</th>
<th>Wavenumber (cm(^{-1}))</th>
<th>Peak assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>unreacted sulbactam</td>
<td>1789</td>
<td>(v(C=O)) of (\beta)-lactam ring</td>
</tr>
<tr>
<td></td>
<td>1402</td>
<td>(v(-CO_2^-)) stretch at C3</td>
</tr>
<tr>
<td></td>
<td>631</td>
<td>(v(-C-S)) between S1 and C5</td>
</tr>
<tr>
<td>Base catalysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulbactam (30 min)</td>
<td>1630</td>
<td>(v(C=N)) of deprotonated imine</td>
</tr>
<tr>
<td></td>
<td>1581</td>
<td>(v(C=C)) of (\text{cis})-enamine</td>
</tr>
<tr>
<td></td>
<td>1402</td>
<td>(v(-CO_2^-)) stretch at C3 and C7</td>
</tr>
<tr>
<td></td>
<td>600/536</td>
<td>(v(-SO_2^-))</td>
</tr>
<tr>
<td>PSR-3-283A (30 min)</td>
<td>1631</td>
<td>(v(C=N)) of deprotonated imine</td>
</tr>
<tr>
<td></td>
<td>1594</td>
<td>(v(C=C)) of (\text{cis})-enamine</td>
</tr>
<tr>
<td></td>
<td>1403</td>
<td>(v(-CO_2^-)) stretch at C3 and C7</td>
</tr>
<tr>
<td></td>
<td>597/538</td>
<td>(v(-SO_2^-))</td>
</tr>
<tr>
<td>SHV-1 catalysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulbactam (23 ms)</td>
<td>1588</td>
<td>(v(C=C)) of (\text{cis})-enamine</td>
</tr>
<tr>
<td></td>
<td>556</td>
<td>(v(-SO_2H)) of (\text{cis})-enamine</td>
</tr>
<tr>
<td>PSR-3-283A (23 ms)</td>
<td>1780</td>
<td>(v(C=O)) of (\beta)-lactam ring</td>
</tr>
<tr>
<td></td>
<td>1630</td>
<td>(v(C=N)) of deprotonated imine</td>
</tr>
<tr>
<td></td>
<td>1612</td>
<td>(v(C=C)) of (\text{trans})-enamine</td>
</tr>
<tr>
<td></td>
<td>1586</td>
<td>(v(C=C)) of (\text{cis})-enamine</td>
</tr>
<tr>
<td></td>
<td>555</td>
<td>(v(-SO_2H)) of enamine</td>
</tr>
</tbody>
</table>
Figure III. 2 Base hydrolysis of sulbactam and PSR-3-283A. 2 µl of 5 mg/ml sulbactam (A) or 5 mg/ml PSR-3-283A (B) were incubated with 2 µl 1 M NaOH at room temperature. The reaction was recorded immediately after the incubation began using Raman spectroscopy.
III.3.4 **Raman studies of reactions with SHV-1 in solution**

In order to compare the inhibition mechanisms of the β-lactamase between sulbactam and PSR-3-283A, we utilized rapid mix - rapid freeze combined with Raman microspectroscopy to monitor the intermediates and products formed in their reaction pathways. Sulbactam or PSR-3-283A were incubated with SHV-1 at the ratio of 1:10 (**E:**I), and the reaction was quenched by liquid pentane immersed in liquid nitrogen at different time points (23 ms - 5 s). The quench temperature was approximately -110 °C. Figure III.3A shows the reaction of SHV-1 with sulbactam (**E:**I = 1:10). The characteristic substrate Raman peak (1789 cm\(^{-1}\)) is assigned to the carbonyl group (C=O) in the β-lactam ring.

In the difference spectrum after mixing, this peak completely disappears at 23 ms indicating all the sulbactam molecules are hydrolyzed by SHV-1. The substrate peaks are replaced mainly by a new peak that is designated as the cis-enamine (1586 cm\(^{-1}\)) in the reaction pathway (species 4, Scheme III.2, Table III.4) (61). This peak originates from the coupled C=C stretch mode in cis-enamine (Table III.4). Both the disappearance of the 631 cm\(^{-1}\) peak (C\(_5\)-S\(_1\) group) and the new profile at 602/556 cm\(^{-1}\), assigned to the -SO\(_2\)H group (since now the pH is ≈ 7.0 and the 600/535 cm\(^{-1}\) profile for –SO\(_2\) are replaced by the 602/555 cm\(^{-1}\) profile for -SO\(_2\)H), indicate the opening of the five-membered ring. The enzyme remained fully active against nitrocefin (a colorimetric substrate of β-lactamase) (111, 112), indicating that the cis-enamine species is hydrolyzed product and there is no acyl-enzyme complex in Figure III.3A.
**Scheme III. 2** The proposed reaction mechanism for PSR-3-283A/sulbactam with SHV-1.

The proposed reaction mechanism for PSR-3-283A/sulbactam with SHV-1 (Scheme III.2) is depicted above. This scheme illustrates the interaction between the antibiotics and the enzyme, showing the formation of various complexes and the regeneration of the active enzyme.

Figure III.3B shows the results for the freeze quenched species formed by the reaction between SHV-1 and PSR-3-283A (E:i = 1:10). Three unresolved peaks (1630, 1612, and 1586 cm⁻¹) at 23 ms are assigned to acyl-enzyme complexes made up of populations of species 3, 5 and 6 in Scheme III.2 although extensive band overlap precludes definitive assignments. In this analysis, the 1630 cm⁻¹ peak is equivalent to the 1632 cm⁻¹ peak in Figure III.2B, which is assigned to the deprotonated imine (-C=N-); peaks at 1612 and 1586 cm⁻¹ correspond to the trans and cis-enamine, respectively (Table III.4).

The appearance of the completed spectral profile at 23 ms spectrum indicates that acylation rate is fast for PSR-3-283A. However, the deacylation rate is much slower than that for sulbactam because the peak at 1780 cm⁻¹ that represents C=O stretch in the intact β-lactam ring is still evident at 23 ms and to a small extent at 5 s. The 1696 cm⁻¹ band seen at 5 s is due to the coupled C=O stretch from the conjugated acryloyl species (-C=C(-O)-O-) in acyl-enzyme complexes 5 and 6 (Scheme III.2) (113, 114). At 5 s growing intensity of the 597/555 cm⁻¹ profile (-SO₂H in species 4, Scheme III.2), as well as the decrease of 1782 cm⁻¹ peak (C=O in β-lactam ring), suggests that several turnovers have occurred and some of the acyl-enzyme complexes at 23 ms are hydrolyzed and released.
as products. At 23 ms, the 597/555 cm$^{-1}$ is of low intensity which reinforces the conclusion that a population of unreacted inhibitor remains. Once product is released, the -SO$_2$H population increases and generates the more intense profile at 597/555 cm$^{-1}$.

However, a substantial population of imine species (1630 cm$^{-1}$, Table III.4) remains at 5 s which is believed to covalently bind to the active site of SHV-1 and inhibit the enzyme.

The presence of a relatively stable acyl enzyme is supported by the unreacted PSR-3-283A peak at 1782 cm$^{-1}$ (Figure III.3B), as well as a nitrocefin assay showing that nitrocefin was not hydrolyzed by the enzyme.

The base hydrolysis data show that interconversion between the imine and enamine product is slow, 20-40 minutes, for both sulbactam and PSR-3-283A. Thus, in Figure III.3A for SHV-1 catalysis of sulbactam the prevalence of cis-enamine product means that very rapid imine-to-enamine interconversion has occurred in the active site, likely catalyzed by E166 (62) followed by hydrolysis of the acyl enzyme. Similarly, for PSR-3-283A the mixture of imine and enamine in the acyl enzyme population is maintained in the product mix indicating that hydrolysis of both acyl enzyme species occurs at similar rates. Obviously, PSR-3-283A makes a more stable acyl enzyme population than sulbactam although it requires a turnover of 80 to block all activity after 24 hours. Again, considering the base hydrolysis data we do not expect enamine to imine interchange to occur when PSR-3-283A product is released from SHV-1.
Figure III. 3 Raman studies of reactions with SHV-1 in solution. The SHV-1 (2 mg/ml) was mixed with sulbactam (A) or PSR-3-283A (B) at the molar ration of 1:10 (E:I). Using the KinTek device, the reaction was quenched at different time points from 23 ms to 1 min. The quenched samples were then freeze-dried and their Raman spectra were recorded.
III.3.5 X-ray and Raman analysis of single crystals

From the kinetic and solution Raman results, we conclude that PSR-3-283A is a more effective inhibitor than sulbactam because its deacylation rate is much slower than that for the sulbactam derived species; it forms the relatively stable inhibited acyl-enzyme complexes that are likely a mixture of imines and enamines. We now provide a rationale from the structural analysis to show how the 6-hydroxymethyl group in PSR-3-283A increases acyl enzyme stability.

Soaking PSR-3-283A into crystals of SHV-1 had a minimal effect on the structure of the enzyme. Superpositioning of all Ca atoms of the inhibitor complex onto the apo \textit{wt} SHV-1 high resolution structure (PDB ID 4FH4) yields a low r.m.s.d. of 0.144 Å indicating that the overall structure was essentially unaffected by inhibitor binding. The acyl region C7-C6-C5-N4 has well defined electron density (Figure III.4A) with the C7-C6-C5-N4 torsion angle being -179.4°. Thus, the C7 and N4 atoms are in the \textit{trans} position. This shows that either the imine (species 3, Scheme III.2) or \textit{trans}-enamine (species 6, Scheme III.2) has been trapped in the active site. The spectroscopic evidence above favors a mix of imine and enamine species. The tail region of PSR-3-283A beyond the carboxylate moiety does not seem well ordered (Figure III.4A). The electron density after the carboxylate is broken although there is strong 6.5σ \textit{F}_o-\textit{F}_c difference density in the vicinity likely representing the electron-dense sulfone moiety of PSR-3-283A.

The inhibitor is covalently bound via its C7 atom to Oγ of S70; the carbonyl oxygen atom of the inhibitor is situated in the oxyanion hole (formed by the backbone nitrogen Hs of residues 70 and 237). The C6 hydroxyl atom has two conformations (Figure III.4B). The main conformation, labeled 1 with 70% occupancy, has the hydroxyl
pointed toward the deacylation water pocket. Crucially, in this position, the hydroxyl moiety hydrogen bonds with the deacylation water and also with E166 and N132 (Figure III.4B). It is known that E166 acts as a general base to hydrolyze the substrate via the interposing deacylation water molecule. Here, the formation of the two hydrogen bonds with the water molecule and E166 may impair the function of E166, hindering it from activating that water molecule to hydrolyze the imine/enamine species formed by PSR-3-283A. It has been proposed that E166 acts as a general base to promote imine to enamine tautomerization (62). The bond between E166's side chain and the -CH₂OH hydroxyl group will also interfere with this function and provides additional evidence as to why transformation from imine to enamine is incomplete for the acyl enzyme formed from 6β-hydroxymethyl sulbactam.

The second conformation of this C6 hydroxyl atom, labeled 2 with 30% occupancy, hydrogen bonds with the backbone oxygen of S130 in one of the conformers of the S130 side chain (Figure III.4B). In addition, one of the oxygen atoms of the sulfone moiety of PSR-3-283A is within hydrogen bonding distance with H (NH₂) of the N170 side chain.

In order to compare the X-ray and Raman data directly, we undertook a single crystal Raman experiment running the reaction by soaking in PSR-3-283A. After PSR-3-283A was soaked in the holding solution containing a SHV-1 single crystal, intense peaks at 1630 cm⁻¹ (imine species in Figures III.2 & 3) and near 1608 cm⁻¹ were detected (Figure III.5). This indicates a mixture of imine and trans-enamine (Table III.4). Considering that there was still unreacted PSR-3-283A present due to nonspecific binding, we performed a soakout experiment. After a SHV-1 single crystal was incubated
with PSR-3-283A for 15 mins, it was transferred to a fresh drop that does not contain any PSR-3-283A. After 15 mins soakout, the unreacted PSR-3-283A was washed away (the 1777 cm$^{-1}$ peak was now absent) but the 1630 and 1607 cm$^{-1}$ peaks remained (Figure III.5), indicating that the imine and trans-enamine species are covalently bound to the active site and inhibit the enzyme.
Figure III. 4 PSR-3-283A bound to SHV-1 active site. A) Stereofigure of electron density $|\text{Fo}| - |\text{Fc}|$ difference density of PSR-3-283A with PSR-3-283A and deacylation water (labeled W) not included in refinement. Electron density is contoured at 3.25σ. Alternate conformations for the 6α-OH moiety are labeled by 1 and 2. B) Interactions of PSR-3-283A in the active site. Hydrogen bonds are depicted as dashed lines with hydrogen bond distances shown. The deacylation water is labeled W.
The Raman data for the SHV-1 single crystal is consonant with the X-ray crystallographic results shown in Figure III.5. The latter indicate that the C6 and C3 atoms in the acyl fragment are \textit{trans} about the C5-N4 bond. The Raman data indicate that these are in fact a mixture of imine and \textit{trans}-enamine present with the heavy atoms in the C6-C5-N4-C3 fragment in the same position. The X-ray data at 1.37 Å resolution are unable to distinguish the tautomers.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{raman_spectra.png}
\caption{Soak-in and soak-out reactions of PSR-3-283A in crystals of SHV-1. 1 μl 20 mM PSR-3-283A was added to 4 μl holding solutions containing a SHV-1 crystal. Raman spectra were recorded at different time points. After 15 min, the crystal was transferred to a fresh drop that did not contain the PSR-3-283A inhibitor. Raman spectra were taken at different time points. The spectra shown in the figure are 15 min after PSR-3-283A was soaked in (middle) and 15 min after the SHV-1 crystal was transferred out to the holding solution (bottom).}
\end{figure}

\textbf{III.3.6 Weak antibiotic activity}

In order to test PSR-3-283A's ability to synergistically help antibiotics kill bacterial cells, disk diffusion assays were conducted and zone diameters were measured for \textit{E. coli} DH10B without or with pBCSK(−) \textit{bla}_{SHV-1} (Table III.3). Interestingly, when
PSR-3-283A was used alone, it also had inhibition against *E. coli* DH10B control cells (12 mm) but not for SHV-1 expressing cells, which indicates that PSR-3-283A also possesses weak antibiotic activity. As an antibiotic, PSR-3-283A should affect the bacterial cell wall formation, such as peptidoglycan synthesis or crosslinking, leading to the cell morphology change or death. After the cells were treated with 10 μg/ml PSR-3-283A for 3 hours, cells were checked under an optical microscope. Compared to the untreated cells, the cell morphology is dramatically changed (Figure III.6A). The cells become longer but cannot divide properly due to the cessation of peptidoglycan synthesis. This change is consistent with the cells treated with methicillin (67). In order to test if PSR-3-283A can react directly with the penicillin binding protein (PBP), we reacted it with PBP5, a penicillin binding protein that affects cell diameter, contour, and morphology in *E. coli* (115). PBP5 and PSR-3-283A were incubated at 1:2 ratio (E:I) in solution and intermediates or products were monitored using the rapid mix - rapid freeze system. In Figure III.6B, the spectrum shows the reaction at 10 s after mixing. The disappearance of the 1773 cm\(^{-1}\) peak indicates that the reaction occurred and no substrate remained. The unresolved peaks at 1623 and 1594 cm\(^{-1}\) show that a mixture of imine and *cis*-enamine are produced based on the above analysis on SHV-1 β-lactamase. Again, it is likely in the cell the imine species is covalently bound to the active site of PBP5 blocking activity and affecting the cell wall morphology. Considering that there is no free PSR-3-283A left, in Figure III.6 *cis*-enamine is likely the hydrolyzed product. PSR-3-283A does not inhibit PBP5 stoichiometrically, which is consistent with its weak antibiotic activity.
**Figure III. 6** PSR-3-283A also possesses weak antibiotic activity. (A) *E. coli* DH10B cell morphology changes upon treated by PSR-3-283A; (B) PBP5 reaction with PSR-3-283A in solution at 1:2 ratio. PSR-3-283A was hydrolyzed by PBP5.

### III.4 DISCUSSION

Using Raman spectroscopy and X-ray crystallography, we are able to elucidate the inhibition mechanism of PSR-3-283A, a 6β-(hydroxymethyl)penicillanic acid sulfone,
against SHV-1 class A β-lactamase. The slow tautomeration and deacylation observed are attributed to H-bonds from the additional 6-hydroxymethyl group.

Much work has been concentrated on the studies of 6-hydroxyalkylpenicillinate derivatives previously. They are found to display robust inhibitory activity against both class A and class C enzymes, such as TEM-1 and AmpC β-lactamases (116, 117). The Mobashery group proposes 6-hydroxyalkylpenicillinates inhibit class A and C β-lactamases depending on the approach direction of the deacylating water molecule (118). 6α-hydroxyalkylpenicillinates can effectively inhibit class A β-lactamases as the water molecule enters from the α direction, while 6β-hydroxyalkylpenicillinates can inhibit class C enzymes because the water molecule approaches from the β direction. Interestingly, PSR-3-283A, a 6β-(hydroxymethyl)penicillinic acid sulfone, inhibits both class A and C enzymes (99). In particular, PSR-3-283A is able to improve the activity of piperacillin in vitro and in vivo against various β-lactamase producing microorganisms (97). The 6α-(hydroxymethyl)penicillinic acid sulfone, on the other hand, did not show any inhibition against class A or C enzymes in the IC₅₀ or MIC assays (unpublished screening work, Bethel and Bonomo). The X-ray structure of SHV-1 complexed with PSR-3-283A shows that the 6-hydroxymethyl group has two positions in the active site of SHV-1. It adopts two directions: one is H-bonded to the deacylation water molecule, the other points away from that water molecule. It is possible that these two directions mimic the positions of the water molecule in class A and C enzymes, respectively.

The presence of 30% conformation 2 (Figure III.4B) in the active site of SHV-1 may contribute to inhibition: the 6-CH₂OH in conformation 2 forms H-bonds with both K73 (weak bond, 3.6 Å, not shown) and S130. These two residues have been shown to
participate in early events in β-lactamase catalysis: the former abstracts a proton from S70 (119) while the latter facilitates the protonation of the β-lactam nitrogen (120). However, the species in conformation 2 may also undergo hydrolysis since the general base, E166, is free to utilize the catalytic water molecule to deacylate the enzyme, releasing the hydrolyzed product as cis-enamine. This outcome is supported by a population of cis-enamine detected by Raman spectroscopy in the solution reaction of SHV-1 with PSR-3-283A (Figure III.3). The cis-enamine appears to be the favored outcome of both base and SHV-1 catalyzed hydrolysis.

Previously, Papp-Wallace et al. (99) detected not only imine and enamine species but also fragmentation and rearrangement in the reaction pathway of PSR-3-283A with TEM-1 and PDC-3, the AmpC of the pathogen Pseudomonas aeruginosa. Under our conditions of reacting SHV-1 with PSR-3-283A, at 23 ms, there appears to be approximately equal amounts of the enamine (species 5/6, Scheme III.2) and imine (species 3, Scheme III.2) intermediates (Figure III.3). The accumulation of the imine indicates that the presence of the 6-hydroxymethyl group affects the tautomerization from imine to enamine. Kalp et al. showed that E166 in the active site of SHV-1 is involved in this tautomerization (62). For imine to tautomerize to enamine, deprotonation at the C6 position is required. In wt SHV-1, deprotonation at C6 of the imine is mediated by E166, via an intervening water molecule. The importance of C6 position is confirmed by the reaction between 6-D,D-sulbactam and SHV-1 (Figure III.S2). Replacement of the hydrogen by deuterium at C6 leads to the accumulation of peak 1630 cm$^{-1}$ (imine species), indicating that the imine-enamine tautomerization is slower due to the isotopic effect. The base hydrolysis of sulbactam also shows a slow transition from imine (1630 cm$^{-1}$, 3
min, Figure III.2) to cis-enamine (1582 cm$^{-1}$, 60 min, Figure III.2). However, in the presence of the SHV-1 enzyme, sulbactam can complete the imine-enamine transition within 23 ms using the E166 and the deacylation water molecule (Figure III.3).

III.5 CONCLUSIONS

In summary, the 6β-hydroxymethyl penicillanic acid sulfone, PSR-3-283A, possesses key features such as enhanced affinity and a slow deacylation rate compared with the clinical inhibitor, sulbactam. The addition of the 6β-hydroxymethyl group in sulbactam not only restores the inhibitory activity of the latter but also extends it to other class A and C β-lactamases. Raman studies in solution and the X-ray structure confirm the presence of a stable imine intermediate that forms H-bonds with a key residue, E166, and a deacylation water molecule. The latter interactions hinder water-mediated deacylation. The characteristic feature in PSR-3-283A is the quasi stable imine, this is different from other effective class A and C β-lactamase inhibitors, such as tazobactam (61) and SA2-13 (55) which predominantly form stable trans-enamine species in the active site. The findings emphasizes the roles of E166 and the deacylation water molecule. To impair the function of the glutamic acid (or analogous residues in other β-lactamases), or to displace or prevent the approach the deacylation water molecule is a critical challenge to the design of novel β-lactamase inhibitors.
IV.1 INTRODUCTION

Antibiotics have been used to treat bacterial infections for decades. Although, initially, β-lactam based penicillins were spectacularly successful, bacteria have relentlessly acquired resistance to a wide range of chemical interventions (19, 84). Consequently, the threat to human health of bacterial resistance to antibiotic therapy can hardly be overstated.

A major source of resistance is the production of two kinds of β-lactamase enzymes by bacteria, serine-β-lactamases and metallo-β-lactamases (MBLs), causing hydrolysis of antibiotics before they can block cell wall synthesis. Here we focus on the MBLs to see how this special class of enzymes perform the rapid hydrolysis, utilizing one or two Zn ions in the active site. As introduced at Chapter I, MBLs are so unique that they are not inhibited by the clinically used inhibitors such as sulbactam, tazobactam and clavulanic acid. However, they are inhibited by metal ion chelators, such as EDTA and o-phenanthroline. But unfortunately, no therapeutic potential has been demonstrated for the chelators.

New Delhi metallo (NDM-1) β-lactamase is a new subclass of the B1 group of MBLs. The NDM-1 gene was characterized initially in an isolate from Sweden that originated in New Delhi, India. NDM-1 shares very little identity with other MBLs. The most closely related species are VIM-1/VIM-2, sharing only 32.4% in sequence identity. However, NDM-1 confers Enterobacteriaceae with nearly complete resistance to all β-
lactam antibiotics including carbapenems (121). Moreover, NDM-1's genes are typically located on readily transferable plasmids that are prone to rearrangement (122) and can lead to plasmid-mediated horizontal gene transfer. In fact, this novel gene has spread rapidly from India throughout human populations on nearly every continent. Because there are no clinical inhibitors effective against this novel β-lactamase, in present clinical practice, NDM-1 positive bacterial infections can only be treated with a few approved antibacterial drugs, including colistin, fluoroquinolones etc, that avoid hydrolysis by NDM-1. So far there is one exception in the β-lactam antibiotic aztreonam, a monobactam, that is poorly hydrolyzed by NDM-1.

For serine β-lactamases, such as SHV-1 and OXA-24, we have already characterized their interactions with inhibitors that undergo major molecular rearrangements in order to block the active site. The approach relies on a covalent bond being formed between the inhibitor and the active site serine. In contrast, metallo β-lactamases hydrolyze lactams by an entirely different mechanism utilizing one or two Zn$^{2+}$ atoms in the active site, and do not form acyl-enzymes (30). This makes the study of catalytic mechanism in NDM-1 difficult. However, recently King et al. (31) solved the structures of NDM-1 complexed with hydrolyzed β-lactams and the inhibitor L-captopril. For the latter the interaction between captopril's sulfate group and Zn1 in NDM-1 prevents the latter from activating the water molecule to hydrolyze the substrate.

Here, we set out to study the drug-enzyme recognition between NDM-1 and aztreonam to investigate further why NDM-1 poorly hydrolyzes the unique monobactam. Furthermore, although a great deal of work in industry and academia has gone into inhibitor-lactamase complexes in vitro, much less is known about the corresponding
reactions inside bacterial cells. Using a new protocol recently developed by our lab, we are able to study the chemical changes of drugs inside bacteria cells, which enables us to compare the reactions \textit{in vitro} and \textit{in vivo} (67). In \textit{toto} this represents a new window on biocidal action that will be used to address fundamental issues in cell-drug interactions.

In our experiments, we unexpectedly found that aztreonam is rapidly hydrolyzed by NDM-1. However, the turnover number is low, indicating that the hydrolyzed aztreonam might have high affinity with the NDM-1 active site and block the active site. After incubation of \textit{E. coli} DH10B \textit{bla}_{NDM-1} cells with aztreonam, a large population of acyl-enzyme complex was detected inside the cells. This might result from the aztreonam reacting with the penicillin binding proteins (PBPs) in the periplasm, indicating that, \textit{in vivo}, aztreonam can overcome the hydrolysis by NDM-1 and particularly target the PBPs, killing the bacterial cells. The results in this chapter are mostly from data obtained recently. Thus, the interpretation of the data is, in some cases, tentative and the conclusions showed here are regarded as preliminary.

**IV.2 MATERIALS AND METHODS**

\textit{Inhibitors}- The antibiotics, aztreonam, erythromycin, ciprofloxacin and rifampicin were purchased from Sigma (St. Louis, MO). 30\% hydrogen peroxide (H$_2$O$_2$) was obtained from Fisher scientific (Waltham, MA).

\textit{Cell lines}- For investigating the reactions between β-lactam antibiotics and cells, \textit{E. coli} DH10B and \textit{E. coli} DH10B \textit{bla}_{NDM-1} were used to compare the reactions in the absence or presence of NDM-1 expression. UCHC \textit{Klebsiella pneumoniae}, a wild type clinical
strain, was a gift from Dr. Amy Anderson, University of Connecticut, and was used to compare with the reactions in *E. coli* cells.

*Expression and purification*—Penicillin binding protein 5 (PBP5, *Pseudomonas aeruginosa*) used in this study was a gift from the Professor Shahriar Mobashery, University of Notre Dame.

To overexpress NDM-1, *bla*<sub>NDM-1</sub> was subcloned into pET24a(+) and then was transformed into *E. coli* BL21 (DE3) cells by Christopher Bethel in Professor Robert Bonomo's lab. Cells were grown in super optimal broth (SOB) supplemented with 50 µg/ml kanamycin at 37 °C until the O.D<sub>600</sub> reaches 0.8. Then 0.2 mM IPTG plus 0.5 mM ZnSO<sub>4</sub> were added to the cultures and cells were grown overnight at 25 °C. Cell pellets were resuspended in 50 ml 50 mM Tris-HCl pH 7.4 containing 500 mM NaCl, 2 mM ZnSO<sub>4</sub>, 0.04 mg/mL lysozyme, 1 mM MgSO<sub>4</sub>, and 5 U/mL benzonuclease. The mixture was stirred for 30 min at room temperature. The mixture was sonicated under the following conditions: 30 s, 50% amplitude, 3 times each sample. The lysate was kept on ice throughout the procedure. The crude extract was centrifuged at 12500 rpm for 20 min, at 4 °C and the supernatant dialyzed versus 2 L of 50 mM Tris-HCl pH 7.4 with 2 mM ZnSO<sub>4</sub>, overnight at 4 °C. The lysate was subsequently purified using HiTrap Q HP column and HiLoad 16/60 Superdex 75 column. NDM-1 β-lactamase was detected using nitrocefin (Calbiochem), a chromogenic β-lactamase substrate.

*Raman studies in solution*—For the rapid mix - rapid freeze experiments, we used a slightly modified KinTek instrument model RQF-3. Reactions were initiated by mixing NDM-1 enzymes (5 mg/ml in 2 mM HEPES, pH 7) with aztreonam in a 1: 10 molar ratio (enzyme: aztreonam). For PBP5, the enzyme: aztreonam molar ratio was 1: 2. The
mixture then traversed through different reaction loops with pre-determined time delay, and the reaction mixture was rapidly frozen by injecting into isopentane at about -110 °C, contained within a tube that was immersed in liquid nitrogen. Isopentane was removed from the ice, and by freeze-drying the frozen material, the lyophilized powder was obtained that constituted the samples for Raman analysis. The powder was examined using Raman microscope (87) (80 mW of 647.1 nm was used as the excitation source and each data set was acquired using 1 × 100 seconds exposure). Based on the reaction loops and exit line volume (that determine the distance of travel for the mixture of enzyme and substrate prior to freezing) and the rpm of the drive motor, we calculated the time of the reaction from mixing to freeze-trapping. The reaction mixture travels 1 to 2 cm in the air after the exit loop to enter the cold isopentane. A schematic picture of rapid mix - rapid freeze has been described previously (54).

Raman studies in cells- The reaction inside the cells was carried out in a manner similar to that previously described (67). Briefly, *E. coli* or *K. pneumoniae* cells were cultured to O.D<sub>600</sub> 0.8. For each time point, 10 ml cell culture was used. Cell pellets were washed with washing buffer containing 67 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.3) and 10 mM MgCl<sub>2</sub>. The cell pellets were then suspended with certain amount of drug solution and incubated for variable time period. Cell pellets were then collected and washed two times with the washing buffer. In the last step, cell pellets were resuspended with water and freeze dried by liquid nitrogen. The lyophilized sample was characterized by Raman spectroscopy. The Raman difference spectrum was obtained by [cells + drug] minus [cells + buffer].

IV.3 RESULTS AND DISCUSSION
IV.3.1 *Base hydrolysis*

In order to better understand the enzyme catalyzed reactions of aztreonam, we followed base hydrolysis of aztreonam by Raman spectroscopy to help us study the rearrangement of aztreonam following attack by Zn-H$_2$O in NDM-1, or Ser-OH in PBP5. Previous studies (108) have shown that base hydrolysis adopts a similar mechanism to that seen in β-lactamase-based catalysis, which utilizes a nucleophilic OH$^-$ to attack the β-lactam ring. The general process can be described in Scheme IV.1.

**Scheme IV. 1** Aztreonam base-hydrolyzed by 1 M NaOH.

![Scheme IV. 1](image)

The major Raman peak assignments for aztreonam molecules are solved based on the quantum mechanical calculations: The carbonyl group at C2 position gives rise to a peak around 1764 cm$^{-1}$ (top spectrum, Figure IV.1), which is the indicator of whether the β-lactam ring is open. It is not intense here because of low aztreonam concentration; The most intense peak at 1601 cm$^{-1}$ is assigned to the stretching motion of the conjugated N5-C6(=O)-C7=N8 fragment, as well as another contribution from C9=C10 stretch. Another intense peak at 1043 cm$^{-1}$ originates from the -SO$_3$H group at the N1 position.
In the base hydrolysis experiment, 2 µl 10 mM aztreonam and 2 µl 1 M NaOH were incubated and the Raman spectra were recorded at variable time periods. In Figure IV.1, at 3 min, two new peaks at 1506 and 1534 cm\(^{-1}\) appear which are due to the hydrolysis product of aztreonam. The β-lactam ring is open, leading to the breakdown of the conjugation between the N5 and C10 groups. Under these circumstances, the 1506 cm\(^{-1}\) is from C9=C10 stretch in the thiazole ring, and the 1532 cm\(^{-1}\), N5-C6(=O) peptide bond. However, there is still an intense peak at 1594 cm\(^{-1}\) from 3 to 20 min (Figure IV.1), indicating that aztreonam is also resistant to base hydrolysis. The 1594 cm\(^{-1}\) peak here is equivalent to the 1601 cm\(^{-1}\) peak in the unreacted aztreonam in DMSO. The shift is due to the solvent changed from hydrophobic to hydrophilic, DMSO to water respectively.

This is dramatically different from the situation in sulbactam hydrolyzed by 1 M NaOH where 20 mM sulbactam were completely hydrolyzed within 3 min (see Chapter II). The resistance to facile hydrolysis in aztreonam may represent a feature in the monobactam which presents a logical pathway for the design of new antibiotics that are not hydrolyzed by metallo β-lactamases (for more evidence see below). Other evidence for the slow hydrolysis is the time-dependent accumulation of the 1067 cm\(^{-1}\) peak, which is tentatively assigned to the deprotonated sulfonate group formed as the β-lactam ring opens.

In summary, the monobactam aztreonam undergoes hydrolysis in the context of high pH, but the hydrolysis rate is much slower than β-lactams fused to five- or six-membered rings. The next step is to follow the hydrolysis in the enzyme environment.
IV.3.2 Aztreonam reactions with NDM-1 in solution

In order to assess the reaction rate between aztreonam and NDM-1, we employed rapid mix - rapid freeze combined with Raman spectroscopy to monitor the early intermediates on the reaction pathway. Aztreonam was incubated with NDM-1 at the ratio of 1:10 (E: S, enzyme: substrate), and the reaction was quenched by liquid nitrogen at different time points (23 - 10 s).

Figure IV.2 shows the two spectra at 23 ms and 10 s. They display similar patterns as in the base hydrolysis (1502 and 1534 cm\(^{-1}\) peaks). However, the new peaks come out at 23 ms indicating that the initial hydrolysis rate is much faster than in base environment. At 10 s, there is still a large population of unreacted aztreonam present, likely as a result of the hydrolyzed product staying in the active site and blocking the
latter. This is consistent with the previous finding and our collaborator's unpublished data (private communication) that aztreonam is poorly hydrolyzed by NDM-1 enzymes.

Figure IV. 2 Raman difference spectra of reactions between aztreonam and NDM-1 in solution.

IV.3.3 Aztreonam reactions with PBP5 in solution

As an effective β-lactam, aztreonam directly targets penicillin binding proteins in the periplasm side (123). To get the spectral profile of the acyl-enzyme complex, aztreonam was reacted with PBP5 in solution at merely 1:2 ratio (E: S). The results is shown in Figure IV.3. Again, the new peaks at 1499 and 1532 cm⁻¹ indicate the β-lactam ring is open due to the attack by the serine hydroxyl group in the active site of PBP5. The new trace at 1691 cm⁻¹ at 10 s is the indicator of acyl-enzyme complex because the ester
bond in Ser-O-C(=O)- usually generates an intense peak between 1700 and 1750 cm\(^{-1}\) (124). The presence of the 1763 cm\(^{-1}\) peak (unreacted aztreonam) also suggests that PBP5 enzymes are completely inactivated even at 1:2 ratio.

In the clinic, aztreonam inhibits mucopeptides synthesis in the bacterial cell wall by mainly targeting PBP3 and then blocking peptidoglycan crosslinking. Here we show that aztreonam also has high affinity with PBP5, another redundant cell-wall synthesizing enzyme in the bacteria. This is not surprising since bacteria usually express 8–9 kinds of PBPs to catalyze transglycosylation and transpeptidation reactions, resulting in the respective polymerization and crosslinking of the glycan strands. These different PBPs have similar overall folding structures and substrate profiles (9).
Figure IV. 3 Raman difference spectra of reactions between aztreonam and penicillin binding protein 5 (PBP5) in solution.

IV.3.4 Aztreonam-PBP reactions in E. coli DH10B empty cells

For drug design, it is vital to identify long-lived drug-enzyme intermediates formed within cells. We have an established record for characterizing β-lactamase-inhibitor intermediates in single crystals and in solution (54, 61, 65). This forms a solid basis for analyzing similar reactions in the cellular environment. Now, we have the ability to identify the intermediates inside cells and monitor the change of the in-cell population in real time. Here, empty cells represent the E. coli cells that do not produce any β-lactamase, which allows us to specifically measure the aztreonam-PBP reactions inside the cells. Thus we set out to follow the reactions in cells to learn how the mechanisms are changed or unchanged compared with in vitro reactions.
To measure the reaction of aztreonam-PBPs inside the cells, using the most recently developed protocol, cells were grown to 0.8 O.D$_{600}$ and harvested from 10 ml of culture by centrifugation. The cell pellets were washed with buffer, and resuspended in 500 μl buffer containing 10 μg/ml aztreonam. After variable incubation periods the mixture was centrifuged. The pellet was washed with buffer again to remove aztreonam molecules that are outside the cells, and then freeze dried. The Raman spectrum was recorded from freeze dried cells, and Raman difference spectrum, [(cells + drug) - (cells alone)], is obtained.

In Figure IV.4, the spectra show the results of *E. coli* DH10B cells treated with aztreonam for 10 and 60 min, respectively. There are three sets of peaks present in the spectrum at 10 min. The first is the unreacted aztreonam inside the cells, evidenced by the 1781 and 1599 cm$^{-1}$ peak, which are from the C=O in the β-lactam ring and the bulky conjugated group N5-...-C10, respectively. However, this peak is not dominant in the spectrum indicating that the
Figure IV. 4 Raman difference spectra of reactions between aztreonam and *E. coli* DH10B cells that do not produce β-lactamases.

Unreacted aztreonam may no longer be the major species inside the cells; the second set are the new peaks from the acyl-enzyme complex upon aztreonam-PBPs interaction. The characteristic peaks such as 1497 and 1543 cm\(^{-1}\), which also exist *in vitro* reactions with NDM-1, PBP5 or base hydrolysis, are from the respective C9=C10 and N5-C6(=O) stretch due to the interrupted conjugation. Another feature is the intense peak at 1700 cm\(^{-1}\), which is from the ester bond in the acyl-enzymes. These evidence supports that aztreonam molecules successfully penetrate the outer membrane and react with the PBPs in the periplasm. However, which PBP has the highest affinity is unknown in cells. Thirdly, there is an unknown set of peaks, such as 1283, 1201 and 770 cm\(^{-1}\). They are
tentatively assigned to the cell metabolism changes because we see the same pattern from other cell treatments with different antibiotics, like DNA synthesis or transcription/translation inhibitors (see below). Moreover, after *E. coli* cells were treated by aztreonam for more than 60 min, most of the cells became elongated due to the dysfunction of peptidoglycan crosslinking. Thus the cells could not divide normally.

Early studies have shown that PBP inhibitors could, to some extent, influence cell division and cause cell elongation or filamentation. For example, PBP1-binding β-lactams are the potent triggers of lysis by activating the peptidoglycan hydrolase, while PBP2 inhibitors alter cell shape and are the least effective autolysin inducers (125, 126). Interestingly, PBP3 inhibitors, like aztreonam, can specifically induce filamentation and therefore affect cell division (125). This involves activation of DNA damage-responsive SOS network of genes, such as *SulA* and *DpiAB* two-component system (127-129). The product of these genes can inhibit the polymerization of FtsZ monomers. FtsZ locates in the inner membrane and acts as a septation trigger and promotes formation of division ring (129). Disruption of DpiAB signaling or knockout of *sulA* leads to enhanced β-lactam lethality (130).

**IV.3.5 Aztreonam-PBP reactions in E. coli DH10B bla<sub>NDM-1</sub> cells**

In order to investigate the fate of aztreonam in cells expressing NDM-1 β-lactamases, we treated *E. coli* DH10B *bla<sub>NDM-1</sub>* cells with 10µg/ml aztreonam using the same protocol as above. We aim to compare the reactions with that in empty cells, to see if aztreonam can be hydrolyzed by NDM-1 inside the cells which leads to a smaller population of acyl-enzyme complexes with PBPs.
Figure IV.5 shows the results that are very similar as that in the *E. coli* empty cells. There are still three sets of peaks in the spectra at 10 and 60 min. The intense peak at 1700 cm\(^{-1}\) suggests that, although in the presence of NDM-1 \(\beta\)-lactamase in the periplasm, aztreonam could still bind to its target PBPs. The only difference is the relative intensity of 1598/1499. In cells expressing NDM-1 enzymes, the relative intensity 1598/1499 is much lower, almost half, than in the empty cells. This indicates that some of the aztreonam molecules undergo hydrolysis inside the cells producing NDM-1 enzymes because the two group experiments use the same amounts of cells and aztreonam.
Figure IV. 5 Raman difference spectra of reactions between aztreonam and *E. coli* DH10B cells that are producing NDM-1 β-lactamases.

Regarding the new "unknown" peaks (e.g. 1281, 1214 and 768 cm\(^{-1}\)), previous studies by the Collins group show a common mechanism of cellular death induced by a wide variety of bactericidal antibiotics that involves the production of reactive oxygen species (ROS), such as hydroxyl radicals (*13I*). A direct outcome of the ROS is the induction of DNA damage. In support of this idea, other reagents or antibiotics (DNA damage inducers or not) were used to treat the *K. pneumoniae* cells to compare if they have the similar effects on the cell metabolism. Four representatives from different families were tested: ciprofloxacin from the quinolone family; rifampin from the transcription inhibitor family; erythromycin from the aminoglycoside family and hydrogen peroxide (H\(_2\)O\(_2\)). Interestingly, when *K. pneumoniae* WT cells (clinical strains)
were treated by 1 µM H₂O₂ for 20 min, the triplet peaks (1283, 1212 and 771 cm⁻¹) again are present (Figure IV.6). H₂O₂ is a known reactive oxygen species that has been implicated as a cause of cell death, mutagenesis and carcinogenesis (132, 133). This is primarily due to its role in inducing DNA damage, such as single or double strands lesions. One of the key mediators is the intracellular iron which reacts with H₂O₂ by a Fenton type of reaction (134). The resulting reactive hydroxyl radical then attacks DNA, causing single-strand breaks (major) and double-strand breaks (minor). We have corroborated this hypothesis by using another DNA-damage inducer. The quinolone ciprofloxacin is a DNA gyrase inhibitor that interferes with DNA replication. The net effect of quinolone treatment is to generate double-stranded DNA breaks and cause replication for arrest because the function of DNA gyrase is compromised. It is interesting that the quinolone family inhibitors also cause filamentation (similar as aztreonam) by activating the SOS response although they do not influence peptidoglycan synthesis directly (135). In Figure IV.6, the spectrum of cell treatment by ciprofloxacin is very similar to that in H₂O₂ treatment, indicating that ciprofloxacin may have similar effect on cells as both H₂O₂ and aztreonam. This should be worthy of attention because it is important to know which effect plays the major role in causing cell death, for example in aztreonam, the direct inhibition of cell wall synthesis or indirect DNA damage?

Recently the Lewis group (136) and the Imlay group separately (137) published their results that cell death from antibiotics is independent of the reactive oxygen species (ROS). High or low concentration of ROS does not correlate with the survival rate of bacteria under aerobic or anaerobic conditions. Of course, this does not preclude the
possibility that antibiotics can induce the SOS response and cause the DNA-damage.

These conclusions are contested by Collins et al. (131) and the field remains very active.

**Figure IV. 6** Raman difference spectra of *K. pneumoniae* cells treated by different classes of reagent or antibiotics: H$_2$O$_2$ - 1 µM, Ciprofloxacin, Erythromycin and Rifampin.
Chapter V: Carboxylation and Decarboxylation of Active Site Lys 84

Controls the Activity of OXA-24 β-Lactamase of Acinetobacter baumannii: Raman Crystallographic and Solution Evidence

ABSTRACT: The class D β-lactamases are characterized by the presence of a carboxylated lysine in the active site that participates in catalysis. Found in Acinetobacter baumannii, OXA-24 is a class D carbapenem hydrolyzing enzyme that exhibits resistance to most available β-lactamase inhibitors. In this study, the reaction between a 6-alkylidenepenam sulfonyl inhibitor, SA-1-204, in single crystals of OXA-24 is followed by Raman microscopy. Details of its reaction with SA-1-204 provide insight into the enzyme’s mode of action and help define the mechanism of inhibition. When the crystal is maintained in HEPES buffer, the reaction is fast, shorter than the time scale of the Raman experiment. However, when the crystal holding solution contains 28% PEG 2000, the reaction is slower and can be recorded by Raman microscopy in real time; the inhibitor’s Raman bands quickly disappear, transient features are seen due to an early intermediate, and, at approximately 2–11 min, new bands appear that are assigned to the late intermediate species. At about 50 min, bands due to all intermediates are replaced by Raman signals of the unreacted inhibitor. The new population remains unchanged indicating (i) that the OXA-24 is no longer active and (ii) that the decarboxylation of Lys84 occurred during the first reaction cycle. Using absorbance spectroscopy, a one-cycle reaction could be carried out in aqueous solution producing inactive OXA-24 as assayed by the chromogenic substrate nitrocefin. However, activity could be restored by reacting aqueous OXA-24 with a large excess of NaHCO3, which recarboxylates Lys84. In contrast, the addition of NaHCO3 was not successful in reactivating OXA-24 in the crystalline state; this is ascribed to the inability to create a concentration of NaHCO3 in large excess over the OXA-24 that is present in the crystal. The finding that inhibitor compounds can inactivate a class D enzyme by promoting decarboxylation of an active site lysine suggests a novel function that could be exploited in inhibitor design.

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V.1 INTRODUCTION

Class D β-lactamases, also known as oxacillinases or OXA enzymes, due to their enhanced capability to hydrolyze oxacillin, can possess carbapenemase or extended-spectrum cephalosporinase activity (138-140), and are important determinants of antimicrobial resistance in Gram-negative pathogens, particularly including *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (11, 36, 37, 141). Both *A. baumannii* and *P. aeruginosa* represent two of the most difficult pathogens to treat as they are often multidrug resistant (MDR) and pose a significant threat to hospitalized patients (142). Class D enzymes are frequently not inhibited by the commercial β-lactamase inhibitors, clavulanic acid, sulbactam, and tazobactam. Unlike class A and C serine β-lactamases, the class D β-lactamases have a characteristic hydrolytic mechanism involving a carboxylated lysine residue, which is believed to function as the catalytic base, both during initial acylation of the active site serine and during hydrolysis of the intermediate acyl-enzyme, as shown in Scheme V.1 (38, 143). While there are examples of carboxylated lysine residues in other enzymes (144, 145), it is unusual for this residue to have a direct mechanistic role. One exception to this rule is the antibiotic sensor domain of the BlaR1 protein of the Gram-positive *Staphylococcus aureus*, which shares 28% sequence identity and high backbone homology with class D β-lactamases (143, 146). Rather than catalyzing the hydrolysis of penicillin like the class D β-lactamases, the BlaR1 sensor forms a stable acyl-enzyme with penicillins, leading to a conformational change in the protein; this relays a signal to the cytoplasm that derepresses the transcription of β-lactamase (146-149).

Scheme V.1.
Scheme V. 1 The reaction pathway for penicillin hydrolyzed by OXA-24 β-lactamase (39).

The carboxylated lysine residue of the class D β-lactamases and the BlaR1 sensor are proposed to be crucial with respect to their differing behavior in the presence of β-lactams (143, 146). As noted by numerous investigators, the carboxy terminus of this residue is positioned analogously to E166 of the class A β-lactamases, and properly positioned to function as the catalytic base for both acylation and deacylation in class D β-lactamases. Lysine of the BlaR1 sensor undergoes decarboxylation subsequent to formation of the acyl-enzyme, thus removing the requisite base from the vicinity of the acylated serine, and leading to a stable acyl-enzyme (146, 148), while in some cases the β-lactamases are able to retain the carboxy group and complete the hydrolytic cycle.

Structural and mechanistic features which promote the formation and stabilization of carboxylysine residues are poorly understood. Therefore, discerning the factors that allow class D β-lactamases to form and maintain a carboxylated lysine, while promoting decarboxylation of the analogous residue in BlaR1 could provide insight leading to design of more effective β-lactamase inhibitors.
In order to understand its chemical properties and potential decarboxylation pathways, a carboxylated lysine can be compared with the hypothetical carbamic acid (H$_2$NCO$_2$H). Carbamic acid is predicted to have a p$\text{Ka}$ of 5.89 (COOH) and -1.22 (protonation of the nitrogen), (Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2012 ACD/Labs), thus verifying the protonation occurs on the oxygen of the more basic, and properly positioned, carboxylate anion, and posing the mechanistic issue of how subsequent decarboxylation (and consequent transfer of the proton from oxygen to nitrogen) may occur. Calculations on the decarboxylation of the carbamic acid molecule indicate that the activation energy can be lowered by 44 kcal/mole by the assistance of one water molecule, as shown in Scheme V.2 (150, 151). Direct transfer of the proton from the (carboxy group) oxygen to the nitrogen, with appropriate geometry for the proton to interact with the nitrogen electron pair, while simultaneously maintaining the amide bond resonance, would involve a four-membered transition state and proton transfer over a distance of approximately 2.72 Å, as shown in Scheme V.2A. The N-HO distance is reduced to 2.29 Å by rotating about the amide CO-N bond to the high energy conformation (Scheme V.2B). By contrast, incorporating assistance from a neighboring water molecule, which could simultaneously provide H-bond donor and acceptor interactions with the carboxylated lysine would involve a six-membered transition state and achieves optimal bond distances from the low energy amide conformation as shown in Scheme V.2C. Bou et al. (39) recently proposed that the reason for the divergent behavior of class D $\beta$-lactamases and BlaR1 is the presence of a suitable water molecule to facilitate loss of CO$_2$ in BlaR1(152) and the absence of a corresponding water in the case of the $\beta$-lactamases, a difference potentially caused by
the positioning of a highly conserved (138) hydrophobic valine (or isoleucine) residue at the β-lactamase position 130 (DBL consensus numbering) and a hydrophilic asparagine residue at corresponding position 109 in BlaR1 as illustrated in Figure V.1 (153).

Scheme V. 2 The addition of one water molecule lowers the activation energy during the decarboxylation process of carbamic acid.

Figure V. 1 Comparison of the active-site carboxyllysine environment of OXA-24 β-lactamase (PDB entry 3G4P) with BlaR1 sensor (PDB entry 1XA1).
In this paper we use Raman spectroscopy to explore the details of the reaction of OXA-24 (139) with a 6-alkylidiene penam sulfone inhibitor, SA-1-204 (Box-V.1). This analysis provides novel insight into the enzyme’s mode of action and shows that (de)carboxylation of Lys84 acts as a catalytic switch (146). Earlier work concluded the reaction between SA-1-204 and OXA-1 or SHV-1 (a class A β-lactamase) in a single crystal occurs slowly in HEPES buffer, forming a non-covalent Michaelis-like complex that is stable in the active site up to one hour before hydrolysis (154). However, new evidence we obtained for SHV-1 (Shanmugam and Carey, unpublished work) shows that the reaction is actually fast in HEPES buffer and is complete in less than 3 minutes. For SHV-1, the reaction can be slowed by the addition of PEG 6000, which allows us to record the reaction including intermediate species using Raman microscopy, which is also the case as shown here for OXA-24. Likely that the reaction is also fast in OXA-1 in the absence of PEG, but slow in the presence.

**Box V.1.** 2’-β-(Phenylacetoxy)-6Z-(α’-pyridylmethylidene) penicillin sulfone, SA-1-204
V.2 MATERIALS AND METHODS

Inhibitors- SA-1-204 was synthesized as described previously (155). A stock solution of the inhibitor at 20 mM in 10 mM HEPES buffer (pH 7.5) was prepared for “soak in” experiments with the protein crystals and for UV-absorbance studies. Potency was verified using the colorimetric β-lactamase substrate nitrocefin (Becton Dickson, λ_{max}=482 nm; ε= 17400 M^{-1}cm^{-1}).

Protein Isolation, Purification, and Crystallization- E. coli BL21 (DE3) cells including pET24 (+) plasmid vector containing blaOXA-24 have been described previously (39). These cells were grown in super optimal broth (SOB) containing 50 µg/ml kanamycin at 37 °C in shaker flasks to achieve an OD_{600} around 0.6-0.8. Then IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to the culture with a final concentration of 0.2 mM and the culture was grown for three more hours. The cells were spun down by centrifugation and frozen at -20 °C. The cultures were warmed back to room temperature and lysed by adding lysozyme and sonicating. The supernatant was collected for further purification. OXA-24 β-lactamase was purified by combination of gel filtration and ion exchange chromatography. A gel filtration HPLC (high pressure liquid chromatography) purification step was performed using a Sephadex Hi Load 26/60 column (GE Healthcare) and elution with 50 mM sodium phosphate buffer (pH 7.4). Ion exchange chromatography was performed using Q sepharose and elution with a NH_{4}HCO_{3} gradient. Concentration of the protein was measured by Bio-Rad's protein assay, and the purity of the enzyme was evaluated by SDS-PAGE. Following purification, the OXA-24 β-lactamases were crystallized using the protocol of Bou et al. (39) Briefly, OXA-24 was concentrated to 6 mg/ml in 10 mM HEPES buffer (pH 7.5). Crystals were grown by the
hanging drop vapor diffusion method in a crystallization solution containing 0.1 M HEPES (pH 7.5), 0.1M sodium acetate and 28% PEG 2000. The crystals were obtained in 4-6 days with the dimensions of 0.12 × 0.12 × 0.08 mm.

**Raman Crystallography**- The Raman microscope system has been described previously (50, 51). A single OXA-24 crystal was transferred from the mother liquor solution to a 4 µL drop of 0.1 M HEPES (pH 7.5), 0.1 M NaOAc and 28% PEG 2000. A 647 nm, 80 mW Kr⁺ laser beam (Innova 70 C, Coherent, Palo Alto, CA) was focused on the protein crystals in the 4 µL hanging drop using the 20× objective of the Raman microscope. During data collection, spectra were acquired for 10 s and 10 accumulations were averaged for each time point. The spectrum of inhibitor SA-1-204 was first taken in the absence or presence of PEG. After obtaining spectra of the apo OXA-24 protein crystals, inhibitors were soaked into the drop to achieve a final volume of 5 µL and a final inhibitor concentration of 5 mM. Spectra were then acquired every 2–3 min after addition of the inhibitors. To obtain difference spectra, an apo β-lactamase spectrum was subtracted from the protein-inhibitor spectra at varying time intervals following addition of inhibitor (Supplemental Text).

\[
\text{Raman difference spectrum} = \text{[protein + inhibitor]} - \text{[protein]}
\]

**Absorbance Studies**- For the NaHCO₃ reactivating experiment in a 500 µL reaction system with or without 28% PEG, OXA-24 was mixed with SA-1-204 in the presence or absence of NaHCO₃. Nitrocefin was used as a chromogenic substrate for OXA-24. The final concentrations were OXA-24 5 µM, SA-1-204 50 µM, nitrocefin 20 µM, NaHCO₃ 100 µM or 100 mM. UV-visible absorbance spectra were taken. Each spectrum included absorbance at wavelengths (λ) from 200 nm to 600 nm. These spectra were recorded at
30 second intervals, with the length of the experiment 30 min. The spectra were saved as individual files grouped by experiment and the spectra shown in Figure V.4 are the average of these.

Calculations- *Ab initio* quantum mechanical calculations were performed on CWRU's cluster facility to predict the Raman spectra of SA-1-204 and model intermediate compounds using Gaussian 03 (156). Calculations were performed at the DFT level using the 6-31+G(d) basis set. DFT calculations were performed with Becke's three parameters hybrid method using the correlation functional of Lee, Yang, and Parr (B3LYP). The vibrations giving rise to the most intense calculated peaks could be visualized using "GaussView", revealing which molecular vibrations contribute to the peaks.

V.3 RESULTS AND DISCUSSION

V.3.1 The presence of PEG can decelerate the OXA-24 β-lactamase reaction with SA-1-204 inhibitor

Previous studies in our lab indicated that the reaction between SA-1-204 (Box-V.1) and OXA-1 in single crystal is slow, with little change occurring in the spectrum of the bound ligand in one hour (154). However, crystallographic studies of several other 6-alkylidene-2'-substituted sulfones, with similar structures to the SA-1-204, reacting with OXA-24 in single crystals show that a quasi-stable bicyclic aromatic late intermediate (Z in Scheme V.3) forms after 6 minute soaks in the presence of PEG 2000 (39).
Scheme V. 3 Proposed mechanism of OXA-24 β-lactamase inhibition by SA-1-204. In SA-1-204, \( R^1 \) is \(-O(C=O)CH_2Ph \) (modified from Bou et al., 2010) (39).

In order to compare the reaction in the presence or absence of PEG, we use Raman microscopy to study the reaction in single crystals in real time. At first sight, the data in Figure V.2A, the time dependence of SA-1-204 reacting in an OXA-24 crystal in HEPES-based holding solution, suggest that no reaction has occurred from 2 minutes to 50 minutes. However, close examination shows that weak peaks occur at 2 minutes like 1440 cm\(^{-1}\) and 1322 cm\(^{-1}\) and then decrease with time. These peaks are assigned to late intermediate species \( Z \) in Scheme V.3 in section b) below. Essentially, we these data suggest that one reaction cycle is complete at 2 minutes (the experimental "dead time" of our Raman approach) leaving a trace of late intermediate which is slowly hydrolyzed with time. At 2 minutes, most active sites are reoccupied by new SA-1-204 but this second molecule of substrate does not react since, as elucidated below, the active site is no longer functional. However, the situation is dramatically different when PEG is added to the holding solution. Figure V.2B shows the time dependent Raman difference
spectrum for the inhibitor SA-1-204 reacting in single crystals of OXA-24 in mother liquor containing 28% PEG 2000. The results show that the reaction now occurs more slowly (Figure V.2B). In the figure, at 2 minutes, the substrate peaks have almost disappeared and new peaks appear. In the presence of PEG, we can see not only the late intermediate peaks like 1443 cm\(^{-1}\), 1339 cm\(^{-1}\) and 1322 cm\(^{-1}\) (assigned below) but also peaks like 1657 cm\(^{-1}\) that decay rapidly and are assigned to an early intermediate X in Scheme V.3 (Section below). This indicates that, at 2 minutes, the reaction is still proceeding whereas we saw, in the absence of PEG, that the first cycle of the reaction was complete at 2 minutes.

The findings for the reaction in single crystals above also find support from the experiment undertaken in solution. We used nitrocefin to monitor the enzyme activity in solution in the presence or absence of PEG 2000 since nitrocefin produces an intense red chromophore in the presence of active β-lactamase. After 50 µM SA-1-204 was incubated with 5 µM OXA-24 in the absence of PEG, the enzyme activity was tested every minute. The result showed that the enzyme was completely inhibited at 1 minute since the nitrocefin was not hydrolyzed and the red chromophore did not appear (157). However, in the presence of PEG, the enzyme was still reactive after 5 minutes since nitrocefin was hydrolyzed and product was formed (157). The enzyme was not completely inhibited until 10 minutes in the presence of PEG because acylation is slowed and active OXA-24 is present until about 10 minutes. *In toto*, the results indicate that *in crystallo* or in solution reaction is occurring quickly in HEPES-based solutions but is slowed markedly in solutions containing PEG.
Possible explanations for why 20-30% PEG can decrease the reaction velocity is that PEG may either slow down the molecular diffusivity or necessary conformational changes in the active site due to increasing the viscosity of the solution. The viscosity generally increase when the concentration or the average weight of PEG increases (158). Earlier work showed that PEG acts as a macroviscogen that can help increase the macroscopic viscosity of solution without slowing down the rate of diffusion of small molecules in the solution (159). Thus, in our experiment, the viscosity (\(\eta\)) of solution in the presence of 28% PEG 2000 \textit{in crystallo} is estimated to be about 9.781 mPas which is nine times the viscosity of water (\(\eta = 1.0045\) mPas) (158). The increase in viscosity of the solution due to the presence of PEG decelerates the reaction by slowing down the conformational changes needed in the active site to form intermediates and products. The slower reaction in the presence of PEG allows us to visualize the progression of the reaction \textit{in crystallo} in real time by use of Raman microscopy.
Figure V. 2 Raman difference spectra of OXA-24 single crystal reaction with SA-1-204 in the absence or presence of PEG 2000. A. Raman difference spectrum of OXA-24 and SA-1-204 at 2 min, 11 min, 18 min, 50 min in the absence of PEG. Control spectrum of unreacted SA-1-204 was first taken in the absence of PEG. After SA-1-204 was soaked in, the spectra were taken at above indicated time points. The reaction is almost complete at 2 min. B. Raman difference spectrum of OXA-24 and SA-1-204 at 2 min, 11 min, 18 min, 50 min in the presence of PEG. Control spectrum of SA-1-204 was first recorded in the presence of PEG. After SA-1-204 was soaked in, the spectra were taken at above indicated time points. The presence of PEG decelerates the OXA-24 β-lactamase reaction with SA-1-204 inhibitor.
V.3.2 The reaction of SA-1-204 in OXA-24 crystals undergoes only one cycle

In the previous section, we propose that SA-1-204 reacts much faster in OXA-24 crystals surrounded by holding solution that does not contain PEG 2000. Under both sets of conditions with or without PEG, the OXA-24 / SA-1-204 reaction goes through just one cycle resembling the reaction in Scheme V.3. We now provide evidence to support this claim. First, we examine the kinetic data for the peaks occurring in the slower reaction in the presence of PEG (Figure V.2B). This helps us assign the non-substrate peaks in Figure V.2B to special features from postulated intermediate seen in Scheme V.3. In turn this allows us to identify peaks in the fast and slow reactions (Figures V.2A and 2B) and show that it is likely that only one reaction cycle has occurred.

In the reaction of SA-1-204 in OXA-24 single crystal in the presence of 28% PEG 2000, the results show that the reaction occurs relatively slowly and appears to go through only one cycle (Figure V.2B). In the figure, at 2 minutes, the substrate peaks have almost disappeared and new peaks appear. The peak at 1695 cm\(^{-1}\) in the spectrum is assigned to the methylenic double bond stretch coupled to the pyridine mode which is expected to change markedly when the β-lactam ring opens and the hybridization at C6 changes (see below). Another characteristic feature is at 1761 cm\(^{-1}\), which originates from the carbonyl (C=O) group of the intact lactam ring (see below). In previous reports of reactions between β-lactamases and inhibitors sulbactam, tazobactam and clavulanic acid, the disappearance of this peak is ascribed to opening of the lactam ring and acylation of the enzyme (53). In the present experiment, the peaks at 1761 and 1695 cm\(^{-1}\) disappear, showing that the reaction is occurring. The peak at 1000 cm\(^{-1}\) corresponds to the phenyl ring mode in SA-1-204 structure which acts as an "internal standard" to monitor the
reaction since it remains unchanged during the reaction (see below). At about 11 minutes (Figure V.2B), the substrate peak at 1695 cm\(^{-1}\) begins to regain intensity probably as a result of product leaving the active site and the substrate reentering. After 11 minutes, the substrate peaks continue to "grow in" reaching a maximum at 50 minutes. Surprisingly the substrate remains in the active site unchanged and does not appear to undergo catalysis.

Based on the structures of substrates similar to SA-1-204 and the constitution of the active site in OXA-24, the mechanism proposed by Bou et al. (39) is taken as a working model as shown in Scheme V.3. To identify whether the proposed mechanism is consistent with the observed Raman spectra, \textit{ab initio} quantum mechanical calculations were used to predict the Raman spectra for some of the species seen in Scheme V.3.

Using the Gaussian program (156), calculations were performed for the free substrate, the early intermediate X and the late intermediate Z (Scheme V.3) since x-ray crystallographic data support their existence. For free substrate SA-1-204 (Table V.1), the peaks at 1761 cm\(^{-1}\) and 1695 cm\(^{-1}\) (Figure V.2B) have been described above (yellow and red regions, respectively in the cartoon), as well as the peak at 1000 cm\(^{-1}\) (green region).

Based on the Gaussian calculations, the other narrow features at 1588 cm\(^{-1}\) and 1570 cm\(^{-1}\) (Figures V.2A and 2B) have contributions from the -SO\(_2\)- group and pyridine group motions (purple and orange regions, respectively in the cartoon). The 1472 cm\(^{-1}\) feature is due to a pyridine mode (orange region) while the 1216 cm\(^{-1}\) mode is from methylenic stretch coupled to the pyridine modes (red region).
Table V. 1 Peak assignment for unreacted SA-1-204 from Gaussian calculations.

![Chemical structure image]

<table>
<thead>
<tr>
<th>Experimental group (cm⁻¹)</th>
<th>Calculated group (cm⁻¹)</th>
<th>Raman intensity (calculated)</th>
<th>Peak assignment</th>
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<tr>
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For the proposed intermediate X (Scheme V.3 and Table V.2), the intense peak at 1658 cm\(^{-1}\) (Figure V.2B) is assigned to an in-plane mode involving pyridine and the skeleton to N4 (red region). The 1447 cm\(^{-1}\) peak derives from the ester group covalently linked to the enzyme (blue region). There are two intense vibrations predicted by the calculation that may be unresolved in the experimental 1658 cm\(^{-1}\) feature (Figure V.2B). Amide I features from \(\alpha\)-helices also appear near 1658 cm\(^{-1}\) (94). However, it is unlikely that the 1658 band is due to changes in \(\alpha\)-helices. These invariably give rise to a feature near 945 cm\(^{-1}\) (160-162) and there is no evidence for a time dependent feature near 945 cm\(^{-1}\) in Figure V.2. In addition, The assignment of the 1658 cm\(^{-1}\) to the protonated imine of intermediate X is further supported by calculations in the imine species in SHV-1 \(\beta\)-lactamase intermediates (61), this has a calculated and experimental band at 1658 cm\(^{-1}\) that is assigned to C=NH\(^{+}\) stretch (61). They are associated with the red region in Table V.2 and strongly support the existence of the intermediate X. On the other hand, the energy-minimized structure of intermediate X derived from the Gaussian calculation (data not shown) closely resembles the product structures for four related compounds in the active site of OXA-24 (39). Thus, it is likely that intermediate X fits within the active site as shown in Figure V.2 of Bou et al. (39) In particular, they showed that the group at the C2 position of LN-1-255, which only differs from SA-1-204 by two -OH groups in the phenyl ring, fits into the structure of the product complex without steric hindrance around the C2 position. Thus, for SA-1-204, the reaction intermediate X can be formed with little or no steric hindrance.
Table V. 2 Peak assignment for proposed early intermediate X from Gaussian calculations.

<table>
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<tr>
<th>Calculation results of early intermediate X</th>
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<tr>
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<td><strong>Green</strong></td>
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For the late intermediate Z (Scheme V.3 and Table V.3) that has also been captured by x-ray studies (39), the peaks at 1550, 1339 and 1322 cm\(^{-1}\) are assigned to modes of the doubly-fused rings (red region). The 1447 cm\(^{-1}\) peak is assigned to the ester group and the 1127 cm\(^{-1}\) peak is localized to the five-membered ring (yellow region). The indolizine double ring moiety gives the most intense Raman signal in the calculation of species Z, consistent with the experimental spectra. However, overall this mode is less intense than the 1658 cm\(^{-1}\) band in species X.

Since the peak at 1000 cm\(^{-1}\) is unchanged during the reaction, \(I_{\text{peak}}/I_{1000}\) can reflect the intensity change of other peaks in real time. Figure V.3 plots the relative intensity of key peaks in the Raman spectra in Figure V.2B as a function of time. The intensity of the substrate peak at 1695 cm\(^{-1}\) decreases quickly as SA-1-204 is soaked in. The peak at 1658 cm\(^{-1}\) increases and decreases faster than other major peaks, indicating it originates from an early intermediate in the reaction. The late intermediate peak at 1322 cm\(^{-1}\) increases and plateaus between 2 minutes and 18 minutes, then declines from 18 minutes to 50 minutes, which indicates the formation of final product that leaves the active site after slowly deacylating. The substrate population declines rapidly over 2 minutes but then increases until at 50 minutes when there appears to be a stable population in the active site. A more extensive set of Raman difference spectra of OXA-24 reaction with SA-1-204 are shown in Supplemental Figure V.1, as well as a more detailed kinetic depiction of intensity changes of the main peaks (Supplemental Figure V.2). From the overall kinetic mechanism, a detailed examination reveals that the reaction follows a very clear order: substrate disappears, intermediate(s) form and leave as the product, substrate reenters without being catalyzed.
Unexpectedly, we find that under the conditions of our reaction in the OXA-24 crystal, the reaction goes through one cycle. This finding is without precedent. Bou et al. (39) point out that in contrast to class A β-lactamase, a hydrolytic water molecule is absent in OXA-24 crystallographic structure, and instead, the key role is played by a carboxylated Lys84 (163); this Lys84 is of central importance in OXA-24 and essential for catalytic activity (39). In earlier work Golemi et al. (38) also shows that at low pH, decarboxylation of the Lys70 (a structurally and mechanistically equivalent residue) leads to the inactivity of the enzyme OXA-10. When the enzyme OXA-10 is regenerated by exposure to bicarbonate, activity is restored and the degree of carboxylation correlates with the degree of recovery of activity (164). Here we propose that the "one cycle" of the reaction is due to the decarboxylation of Lys84 in the active site after the first reaction cycle and that in the crystal environment recarboxylation does not occur (Scheme V.3). Support for a one cycle reaction leading to the an inactive enzyme is found in the solution studies described in next section.
Figure V.3 Kinetic depiction of the substrate peak 1695 cm\(^{-1}\), early intermediate peak 1658 cm\(^{-1}\) and late intermediate peak 1322 cm\(^{-1}\) intensities over time.
Table V. 3 Peak assignment for proposed late intermediate Z from Gaussian calculations.

![Diagram of a chemical structure](image)

Calculation results of *late intermediate Z*

<table>
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<tr>
<th>Experimental group (cm⁻¹)</th>
<th>Calculation group (cm⁻¹)</th>
<th>Raman intensity (calculated)</th>
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<tr>
<td>1000</td>
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V.3.3 Recarboxylation of the lysine residue 84 in the active site restores OXA-24's hydrolytic activity towards nitrocefin in solution

The results discussed above indicate that OXA-24 in the crystal undergoes one catalytic cycle and then becomes inactive. A possible explanation lies in the role of carboxylated lysine 84 residue in the catalytic process. All class D β-lactamases characterized to date have a carboxylated lysine residue in the active site (38, 165-167). In OXA-10 class D β-lactamase, carboxylation is a reversible process (38). The lysine spontaneously decarboxylates at low pH 4.5 but can be recarboxylated by exposure to 20 mM NaHCO₃ at pH 7.5. Golemi et al. (38) also show that the hydrolysis of most substrates by OXA-10 is characterized by biphasic kinetics due to the presence of an equilibrium between the uncarboxylated and carboxylated forms of Lys70 (see above) (164). OXA-24 class D β-lactamase also has a carboxylated Lys84 in its active site (163). Thus, we undertook experiments in solution and in single crystals to test our hypothesis that, in the presence of SA-1-204, Lys84 in OXA-24 becomes decarboxylated during the first cycle of catalysis rendering the enzyme inactive.

In solution, 5 µM OXA-24 was reacted with 20 µM nitrocefin. Nitrocefin was used as a chromogenic substrate to monitor the enzyme activity in solution. Almost all the nitrocefin is transformed into product as evidence in the 482 nm absorbance in Figure V.4 (black line). Next, 5 µM OXA-24 and 50 µM SA-1-204 were mixed first for 1 minute and then 20 µM nitrocefin was added. This gives rise to the absorbance peak near 380 nm in Figure V.4 (red line). A nitrocefin product peak is not visible near 482 nm indicating that SA-1-204 completely blocks the activity of OXA-24 towards nitrocefin. However, when the solution of inhibited OXA-24 (with SA-1-204 and nitrocefin) is
treated with 100 μM or 100 mM sodium bicarbonate (NaHCO₃), activity is restored and nitrocefin product appears (green and blue lines, respectively). In a control, there was no effect when 100 mM NaHSO₄ replaced NaHCO₃ (cyan line). The same experiment was also repeated in the presence of 28% PEG 2000. Not only could bicarbonate reactivate the OXA-24 enzyme but also PEG could slow down both the decarboxylation and recarboxylation process (data not shown). In addition, UV-absorbance assays show that the reaction between SA-1-204 and OXA-24 is strictly stoichiometric. While 20 μM OXA-24 and 60 μM SA-1-204 are reacted, the spectrum shows only one third of the SA-1-204 (equivalent to the concentration of OXA-24) is hydrolyzed, indicating that the reaction undergoes only one cycle under these conditions (Supplemental Figure V.3A). But in the presence of NaHCO₃, the enzyme continues hydrolyzing SA-1-204 until the latter is exhausted (Supplemental Figure V.3B). This suggests that the enzyme becomes decarboxylated after the first reaction cycle but can be reactivated by the addition of carbon dioxide generated from bicarbonate.
**Figure V. 4** Recarboxylation of the lysine residue 84 in the active site can restore the OXA-24 hydrolytic activity towards nitrocefin. Black line: OXA-24 was 5 µM in HEPES buffer (10 mM, pH 7.5), then 20 µM NCF was added and the UV-absorbance spectrum was recorded; Red line: reagents were added in the order of 5 µM OXA-24, 50 µM SA-1-204, 20 µM NCF at the intervals of 1 min, then the UV-absorbance spectrum was recorded; Green line: reagents were added in the order of OXA-24, SA-1-204, NCF, 6 and 100 µM sodium bicarbonate, then UV-absorbance spectrum were recorded every 30 seconds in the next 30 minutes; Blue line: the same as in the Green line except that the sodium bicarbonate concentration was 100 mM; Cyan line: the same as in the Green line except using 100 mM sodium bisulfate.

To probe the reaction in a single crystal, a OXA-24 single crystal (Figure V.5A) is transferred into a fresh hanging drop made up of 0.1 M HEPES (pH 7.5). The concentration of active sites is calculated as 15 mM (Supplemental text). Nitrocefin is injected into the drop to give a final concentration of 1 mM. Nitrocefin is hydrolyzed quickly within the crystal since the crystal turns red immediately (Figure V.5B). When 5 mM SA-1-204 is first soaked into the drop containing a OXA-24 single crystal, no
nitrocefin product is generated and the crystal remains yellow (Figure V.5C). When the mixture of above OXA-24 single crystal, SA-1-204 and unreacted nitrocefin is treated with 100 mM NaHCO\textsubscript{3}, nitrocefin product is still not formed and crystal does not turn red (Figure V.5D), indicating that enzyme remains inactive. Thus, while the “in solution” data the one cycle reaction is a consequence of the lysine decarboxylation that can be rescued by the addition of NaHCO\textsubscript{3}, activity could not be restored in single crystal. An explanation is that there is an equilibrium between the uncarboxylated and carboxylated Lys\textsubscript{84}. In the single crystal reaction, the concentration ratio between NaHCO\textsubscript{3} and decarboxylated Lys\textsubscript{84} is about 7:1 since the protein concentration is about 15 mM (Supplemental Text) (47), much lower than that in solution 1000 : 1, so that the Lys\textsubscript{84} remains non-carboxylated and cannot hydrolyze the next coming nitrocefin.

Experimental evidence to date has established that the carboxylated lysine residue results from a reaction between the non-protonated ε-amino group of the lysine and carbon dioxide (150). Studies on OXA-10 reveals that Val\textsubscript{117}, Phe\textsubscript{120} and Trp\textsubscript{154} in the active site maintain a hydrophobic environment (hydrophobic pocket) for the Lys\textsubscript{70}, which decreases the pK\textsubscript{a} of Lys\textsubscript{70}, favoring its reaction with carbon dioxide (166, 168). Crystallographic studies on the active site of OXA-24 indicate that Lys\textsubscript{84} is also in a hydrophobic environment, which consists of Val\textsubscript{130} and Trp\textsubscript{167}. Specifically, the residue Trp\textsubscript{167} interacts with the carboxylate group of Lys\textsubscript{84} and help it locate in an “optimum orientation”. This will also lead to the decrease the pK\textsubscript{a} of Lys\textsubscript{84}, which yields a major proportion of non-protonated ε-amino at physiological pH, favoring its carboxylation (38, 45, 168). The \textit{in vivo} concentration of CO\textsubscript{2} has been reported at 1.3 mM (169); Golemi \textit{et al.} (38) reported that the dissociation constant (Kd) for CO\textsubscript{2} and
monomeric OXA-10 β-lactamase was 12.4 ± 0.01 µM. Considering that OXA-24 β-lactamase is also a monomer, this enzyme is expected to be fully carboxylated in its native state.

![Images of crystal structures](Image)

**Figure V. 5** Low ratio of concentration between NaHCO₃ and OXA-24 (C_{NaHCO₃} : C_{OXA-24}) is unable to reactivate the enzyme. Single OXA-24 crystal was transferred from the mother liquor solution to a 4 µL drop of 0.1 M HEPES (pH 7.5), 0.1 M NaAc and 28% PEG 2000. SA-1-204, nitrocefin and NaHCO₃ were added to test the hydrolysis activity of the crystal. A. OXA-24 crystal in the mother liquor solution. B. 1 µL nitrocefin (1 mg/mL) was added into the solution containing the single OXA-24 crystal. C. 1 µL SA-1-204 (20 mM) was soaked in the single crystal solution for 1 min, then nitrocefin was added. D. 1 µL NaHCO₃ (100 mM) was added into the solution after the SA-1-204 and nitrocefin mixture.

Regarding the decarboxylative mechanism, loss of CO₂ directly from the negatively charged conjugate base of the carbamic acid would involve formation of a nitrogen anion. Given the high pKₐ (ca.35) of such an unstabilized nitrogen anion, it is logical that decarboxylation of the carbamic acid occurs with concurrent transfer of a proton from oxygen to nitrogen possibly through an intermediate water molecule,
predicted theoretically and shown in Scheme V.2. This is consistent with the observation that decarboxylation of the lysine also occurs at pH < 4.5. Thus, for continued β-lactam hydrolysis and avoidance of decarboxylation of the carboxylated lysine, it is important for the enzyme to maintain the anionic conjugate base state of the carbamic acid moiety. 

In the normal hydrolytic sequence, each time the N-carboxy group abstracts a proton, it is rapidly removed, firstly ferrying a proton from the active site serine to the departing N4 as shown in Scheme V.1, B to C, (in order to assist departure of that nitrogen from the tetrahedral intermediate and prevent it from assuming a formal negative charge) and secondly from the hydrolytic water to the same nitrogen, this time the nitrogen acting as a simple amine base (pK_a 10-11), but nonetheless leaving the carbamic acid as its conjugate base to resume the catalytic cycle (Scheme V.1, E to F). With the pyridylalkylidene inhibitor, however, two events interfere with this course of events: 1) the fragmentation of the dioxothiazoline ring system, with ejection of the sulfinate anion, and concomitant intramolecular capture of the resultant imine by the pyridine nitrogen, has now created a positively charged pyridinium ion in Y (Scheme V.3), which can form an aromatic indolizine system by loss of a proton from C5 (penam numbering) to the conjugate base of the carbamic acid and placing it in a (conjugate acid) state more susceptible to decarboxylation (Scheme V.3, Y to Z); 2) In the subsequently formed aromatic intermediate Z, the (penam) N4 atom is now part of a β-aminoacrylate moiety, and thus has reduced pK_a (ca. 5), now insufficient to deprotonate the carbamic acid (pK_a = 6), and thus rendering the carboxyllysine susceptible to decarboxylation. Once this decarboxylation occurs, the resultant amine of the lysine is too distant from the ester linkage of the acyl-enzyme to efficiently function as a base during the hydrolytic process.
Even if the ester is slowly hydrolyzed, without a basic (thiazolidine) nitrogen to carry away the proton from the site, the lysine is left protonated, and unable to react with CO$_2$ in a carboxylative process or to function as a catalytic base to initiate acylation.

In the case of the class A $\beta$-lactamases, the hydrolytic water molecule is well ordered and observed crystallographically. By contrast, an active-site water molecule has never been observed for any class D $\beta$-lactamase (167). Since water is required for hydrolysis of the acyl-enzyme, it is logical to propose that the hydrolytic water molecule is more disordered in class D, as they have large and more hydrophobic active sites.

Bou et al. had earlier proposed that 6-alkyldenepeicillin sulfone inhibitors, which rearrange to bulky, conformationally rigid indolizines subsequent to acylation of the active site serine, may function by displacing the hydrolytic water (39). The present work seems to imply that, although water is displaced from a position in which it could hydrolyze the acyl-enzyme, water is still present proximal to the carboxylated lysine and is capable of facilitating its decarboxylation, as shown in Scheme V.2. It is not yet clear if the water molecule which facilitates decarboxylation of the active site lysine is the same water molecule that is responsible for hydrolysis of the acyl-enzyme. Perhaps the bulky inhibitor has moved the water molecule from a position where it interacts with the terminus of the carboxylated lysine and the active site serine, to a position where it simultaneously interacts with the protonated carbamic acid and the $\varepsilon$-nitrogen of the lysine, thus facilitating decarboxylation as shown in Scheme V.2.
V.4 CONCLUSIONS

In summary, the results of these experiments and the underlying mechanistic explanations provide unique insights that lead us further along the path to understanding on a very fundamental level the reaction chemistry and mechanism of inhibition of these clinically important class D OXA carbapenemases. As the pathogen harboring this β-lactamase (A. baumannii) has become a major threat to our antimicrobial armamentarium, new chemical approaches are desperately needed. The importance of the single turnover reaction cannot be overstated. In effect, we demonstrate that under the appropriate conditions, an OXA-24 β-lactamase is "trapped" and becomes catalytically incompetent as a result of a single turnover interaction with an inhibitor. This unprecedented observation has significant ramifications. The challenge here will be to capitalize on this finding and create novel classes of compounds that accelerate decarboxylation and retard the repopulation of Lys84 by carboxylate. This unique strategy creates an entirely new goal in synthetic chemistry that may be approached using both β-lactam scaffolds that preserve our existing drugs as well as by fragment-based libraries (170). In the latter case, diverse chemotypes may be exploited to achieve single turnover kinetics.
Chapter VI: The Different Inhibition Mechanisms of OXA-1 and OXA-24 β-Lactamases Are Determined by the Stability of Active-Site Carboxylated Lysine

ABSTRACT: The catalytic efficiency of class D β-lactamases depends critically on an unusual carboxylated lysine as the general base residue for both the acylation and deacylation steps of the enzyme. Microbiological and biochemical studies on the class D β-lactamases OXA-1 and OXA-24 showed that the two enzymes behave differently when reacting with two 6-methylidene penems (penem 1 and penem 3): the penems are good inhibitors of OXA-1 but act more like substrates for OXA-24. UV difference and Raman spectroscopy revealed that the respective reaction mechanisms are different. The penems form an unusual intermediate, a 1,4-thiazepine derivative in OXA-1, and undergo deacylation followed by the decarboxylation of Lys-70, rendering OXA-1 inactive. This inactivation could not be reversed by the addition of 100 mM NaHCO3. In OXA-24, under mild conditions (enzyme: inhibitor, 1: 4), only hydrolyzed products were detected, and the enzyme remained active. However, under harsh conditions (enzyme: inhibitor, 1: 2000), OXA-24 was inhibited via decarboxylation of Lys-84; however, the enzyme could be reactivated by the addition of 100 mM NaHCO3. We conclude that OXA-24 not only decarboxylates with difficulty but also recarboxylates with ease; in contrast, OXA-1 decarboxylates easily but recarboxylates with difficulty. Structural analysis of the active site indicates that a crystallographic water molecule may play an important role in carboxylation in OXA-24 (an analogous water molecule is not found in OXA-1), supporting the suggestion that a water molecule in the active site of OXA-24 can lower the energy barrier for carboxylation significantly.

VI.1 INTRODUCTION

\(\beta\)-Lactamase production is the most important mechanism by which Gram-negative pathogens including *Acinetobacter baumannii* and *Pseudomonas aeruginosa* become resistant to \(\beta\)-lactam antibiotics. Based on their protein sequence similarities, they are divided into four major classes (Class A-D) ($^{18}$). Class A, C and D enzymes involve an active-site serine to hydrolyze \(\beta\)-lactams, whereas class B enzymes are zinc-dependent hydrolases. Unlike the majority of class A enzymes which have been extensively studied, class D \(\beta\)-lactamases confer a higher-level resistance to a broad spectrum of \(\beta\)-lactam inhibitors and are the least understood class ($^{138, 167}$).

The class D \(\beta\)-lactamases are characterized by the presence of a unique carboxylated lysine in the active site that participates in catalysis. While carboxylated lysine has also been found in other enzymes, such as rubisco ($^{171}$), urease ($^{172}$) and phosphotriesterase ($^{173}$), there it mainly serves in a structural role. The carboxylated lysine in class D enzymes plays a similar role as the general base (Glu166) in SHV-1 class A \(\beta\)-lactamase ($^{174}$). The formation of the carboxylated lysine is reversible ($^{38}$). Low pH or mutation of hydrophobic residues surrounding the carboxylated lysine, such as Val117 (OXA-1) or Trp154 (OXA-10) results in decarboxylation of that lysine and loss of enzyme's activity, notably deacylation ($^{175-178}$); while the addition of bicarbonate can reactivate the enzyme by recarboxylation of the lysine ($^{38, 178, 179}$).

OXA-1 and OXA-24 are two class D enzymes exhibiting resistance to the clinically available \(\beta\)-lactamase inhibitors (tazobactam, sulbactam and clavulanate). They are both monomeric and are related based on three aspects: 1) ~30% sequence homology; 2) similar folded structures; 3) highly conserved active-site residues (>95%) (Using PDB
entry: OXA-1, 1M6K (167); OXA-24, 3G4P (39)). However, they show differing affinities for β-lactam-based inhibitors. OXA-1 is the most common of the class D β-lactamases and is found in up to 10% of *Escherichia coli* and *Pseudomonas aeruginosa* (167, 180). Its closely-related variants (*e.g.* OXA-15, OXA-18, OXA-19), due to point mutations and plasmid transfer, have arisen with enhanced capability to hydrolyze imipenem, aztreonam and third-generation cephalosporins such as cefotaxime and ceftriaxone (180, 181). Found in *Acinetobacter baumannii*, OXA-24 is a class D carbapenem-hydrolyzing enzyme that also possesses extended-spectrum cephalosporinase activity (138, 140). Both OXA-1 and OXA-24 cause serious problems in nosocomial infections such as bloodstream infections, wound infections, and ventilator-associated pneumonia (139, 182). Thus, the need to develop potent inhibitors of these enzymes is an urgent priority; to achieve this, it is imperative to understand the properties of these enzymes and how they work.

In this chapter, the catalytic properties of OXA-1 and OXA-24 are evaluated using two methyldiene penems (penems 1 and 3, Figure VI.1). Bethel *et al.* in 2008 proposed that penems inactivate OXA-1 β-lactamase efficiently by forming an unusual acyl-enzyme complex (183). Here, our results show that penem inhibitors have a high affinity for both OXA-1 and OXA-24 enzymes. However, they are effective inhibitors of OXA-1 but act more like substrates for OXA-24. UV difference (UVD) and Raman spectroscopies show the reaction pathways are different when penems react with OXA-1 or OXA-24. Existing structural analysis of the active site indicates a crystallographic water molecule may play an important role in carboxylation in OXA-24 (an analogous water molecule is not found in OXA-1), providing further support for the computational
model by Schlegel and Mobashery's groups who showed that a water molecule in the OXA-10 active site can lower the energy barrier for carboxylation (150).

![Chemical structures of inhibitors](image)

**Figure VI.1** Chemical structures of penem inhibitors (penem 1 and penem 3), comparators (tazobactam and BRL 42715), and penam sulfone inhibitor (SA-1-204).

**VI.2 MATERIALS AND METHODS**

*Genetic constructs and host strains*- The $\text{bla}_{\text{OXA-1}}$ gene was cloned from plasmid R$_{\text{GN238}}$ into pET 12a(+) - KM as described previously (167). Plasmid R$_{\text{GN238}}^{\text{bla}_{\text{OXA-1}}}$ was maintained in *E. coli* DH10B cells (Invitrogen, Carlsbad, CA). This host strain was used for minimum inhibitory concentration (MIC) determinations. For protein purification,
*bla*<sub>OXA-1</sub> was cloned in the modified vector pET 12a(+)-KM described previously, and was expressed in *E. coli* BL21(DE3) cells (Stratagene, La Jolla, CA) (181).

For large-scale protein expression and β-lactamase characterization, the *bla*<sub>OXA-24</sub> gene was cloned into the pET24a (+) vector (Novagen, Madison, WI) according to the following method. Using the Gene-Amp XL PCR kit (Applied Biosystems), high-fidelity amplification of *bla*<sub>OXA-24</sub> without leader peptide sequence from the OXA-24/pIM-1-RA clone designed by Héritier *et al.* (12) was performed with primers OXA-24FOR and OXA-24REV, listed in Table VI.1. The cycling conditions used were 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min for 25 cycles, after which there was final extension at 72 °C for 10 min. A restriction digest of the pET24a (+) vector was done using NdeI and BamHI. The amplification product was purified using the QIAquick gel extraction kit (Venlo, Netherlands) and digested using NdeI and BamHI. This product was ligated to the digested pET24a (+) vector and electroporated into *E. coli* DH10B. The resulting construct was sequenced with pET24a (+) primers T7 promoter primer and T7 terminator primer. After sequencing verification, the construct was transformed into *E. coli* BL21(DE3) cells for protein expression.

For MIC determinations, *bla*<sub>OXA-24</sub> was cloned into the pBC SK (+) phagemid vector (Stratagene, La Jolla, CA) as described previously (184). *bla*<sub>OXA-24</sub>/pBC SK (+) sequence was verified using M13 Universal and M13 Reverse primers.
Table VI. 1 Primer sequences.

<table>
<thead>
<tr>
<th>Function and primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
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<tbody>
<tr>
<td>Cloning primers</td>
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<tr>
<td>OXA-24FOR</td>
<td>CATATGCTCTATAAAAACTAAATCTGA</td>
</tr>
<tr>
<td>OXA-24REV</td>
<td>GGATCCTTAATGATTCCAAGA</td>
</tr>
<tr>
<td>OXA-24LDR</td>
<td>CATATGAAAAAATTTATACCTTCCTATATTC</td>
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Sequencing (Cy5-labeled) primers

<p>| | |</p>
<table>
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<th></th>
</tr>
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<tbody>
<tr>
<td>M13 Universal</td>
<td>GTAAAACGACGGCCAG</td>
</tr>
<tr>
<td>M13 Reverse</td>
<td>CAGGAAACAGCTATGAC</td>
</tr>
<tr>
<td>T7 Promoter</td>
<td>TAATACGACTCATAAGGG</td>
</tr>
<tr>
<td>T7 Terminator</td>
<td>GCTAGTTATGCTACGCGG</td>
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</table>

Inhibitors - The inhibitors penem 1 and penem 3 (Figure VI.1) were gifts from Wyeth Pharmaceuticals (Madison, NJ). Their chemical synthesis were described previously (185). A stock solution of the inhibitor at 20 mM in 10 mM HEPES buffer (pH 7.5) was prepared for "soak in" and "soak out" experiments with the protein crystals and for UV-absorbance studies. Only water was used in the rapid mix-rapid freeze experiment to avoid the interference of salt signal. Potency was verified using the colorimetric β-lactamase substrate nitrocefin (Becton Dickson, \( \lambda_{\text{max}} = 482 \text{ nm}; \varepsilon = 17,400 \text{ M}^{-1} \text{ cm}^{-1} \)).

Antibiotic Susceptibility - The MICs of E. coli DH10B expressing bla_{OXA-1} or bla_{OXA-24} β-lactamases were determined in Mueller-Hinton (MH) agar supplemented with 20 mM NaHCO₃ using a Steers replicator which delivered 10 µl of MH broth containing \( 10^4 \) colony-forming units/spot. The penem inhibitors were tested at the concentration of 4 µg/ml partnered with piperacillin (Sigma, St. Louis, MO) at concentrations of 1-2048
μg/ml. As a comparator inhibitor, tazobactam (Chem-Impex, Wood Dale, IL) was used at the concentration of 4 μg/ml. Breakpoints for susceptibility and resistance were defined by the Clinical Laboratory Standards Institute and interpreted with criteria published in 2005 (CLSI standard M100-S15) (102, 186).

**Protein Isolation, Purification, and Crystallization**- OXA-1 or OXA-24 were purified as described previously (65, 183). Concentration of the protein was measured by Bio-Rad's protein assay, and the purity of the enzyme was evaluated by SDS-PAGE. Following purification, OXA-1 β-lactamase was crystallized using the protocol described in Sun et al. with protein concentration of 9 mg/ml (167). Crystals were grown by the hanging drop vapor diffusion method in a crystallization solution containing 0.05 M HEPES (pH 7.5) and 15% PEG 8000. OXA-24 β-lactamase was crystallized using the protocol of Bou et al (39). Briefly, OXA-24 was concentrated to 6 mg/ml in 10 mM HEPES buffer (pH 7.5). Crystals were grown by the hanging drop vapor diffusion method in a crystallization solution containing 0.1 M HEPES (pH 7.5), 0.1 M sodium acetate and 28% PEG 2000.

**Ultraviolet difference (UVD) spectroscopy**- In our protocol, each spectrum included absorbance at wavelengths (λ) from 200 nm to 600 nm. The spectrum of only β-lactamase (20 μM) or inhibitor (20 or 80 μM) was taken separately. Then OXA-1 and OXA-24 (20 μM for both) reacted with penem 1 (20 or 80 μM) in a ratio of 1: 1 or 1: 4 (E: I) and UV-absorbance spectra were taken at 30 second intervals, with the length of the experiment being 30 min. To obtain UV difference spectra, an apo β-lactamase spectrum was subtracted from the protein-inhibitor complex spectra at varying time intervals following addition of inhibitor. Nitrocefin (NCF) assay uses nitrocefin as a chromogenic substrate to monitor the enzyme activity in solution. When OXA-1 or OXA-
24 react with NCF, NCF is hydrolyzed and turns red ($\lambda_{\text{max}} = 482$ nm); when the enzyme is inhibited, NCF is unreacted and remains yellow ($\lambda_{\text{max}} = 382$ nm).

**Raman Spectroscopy**- The Raman microscope system has been described previously (50, 51). A single OXA-1 or OXA-24 crystal was transferred from the mother liquor solution to a 4 µl drop of 0.05 M HEPES (pH 7.5) and 15% PEG 8000 (for OXA-1) or a drop of 0.1 M HEPES (pH 7.5), 0.1 M NaOAc and 28% PEG 2000 (for OXA-24). After obtaining spectra of the apo protein crystals, inhibitors were soaked into the drop to achieve a final volume of 5 µl and a final inhibitor concentration of 5 mM. Spectra were then acquired every 2–3 min after addition of the inhibitors. To obtain difference spectra, an apo β-lactamase spectrum was subtracted from the protein-inhibitor spectra at varying time intervals following addition of inhibitor (65).

To study the reaction in solution at early time points, we used a slightly modified KinTek instrument model RQF-3 (54). Reactions were initiated by mixing OXA-1 or OXA-24 enzyme (2.5 mg/ml) with penem inhibitors in a 1: 2 molar ratio and quenched at 1 s. The sample after reactions was examined using Raman microscope (87). For longer time scale reaction, we also used hand-mixing system which incubated enzyme with inhibitor at 1: 2 ratio for 5 s and 30 s and then quenched the reaction by injecting into the isopentane solution surrounded by liquid nitrogen.

**Kinetics**- Steady state kinetics were performed on an Agilent 8453 diode array spectrophotometer (Palo Alto, CA) in 50 mM Na phosphate buffer (pH 7.2) supplemented with 20 mM NaHCO$_3$. $V_{\text{max}}$ and $K_m$ were determined from initial steady-state velocities for nitrocefin (NCF). The kinetic parameters were obtained using
iterative non-linear least-squares fit of the data to the Henri-Michaelis equation using Origin 8.1 (OriginLab, Northampton, MA) according to Equation 1:

\[ v = \frac{V_{\text{max}} [S]}{(K_m + [S])} \] (1)

The overall mechanism for β-lactamase-inhibitor reaction is shown in Scheme VI.1. We determined the \( K_i \) for the penems by measuring initial steady-state velocities in the presence of a constant concentration of enzyme with increasing concentrations of inhibitor against the indicator substrate NCF (100 µM). Assuming a competitive mode of inhibition under these conditions, initial velocity \( (v_0) \) measurements immediately after mixing yield a \( K_i \) which closely approximates \( K_m \), as represented by Equation 2:

\[ v_0 = \frac{(V_{\text{max}}[S])}{K_m(1 + K_i) + [S]} \] (2)

\( K_i \) values were corrected for nitrocefin affinity (\( K_m = 8.3 \) µM for OXA-1, \( K_m = 28 \) µM for OXA-24) according to Equation 3:

\[ K_i(\text{corrected}) = \frac{K_i(\text{observed})}{(1 + [\text{NCF}]/K_{m\text{NCF}})} \] (3)

IC\(_{50}\), defined as the inhibitor concentration resulting in a reduction of NCF (100 µM) hydrolysis by 50%, was determined by measurements of initial velocities after 5 min pre-incubation of enzyme with inhibitor.

Turnover numbers \( (t_n) \) or partition ratios \( (k_{\text{cat}}/k_{\text{inact}}) \) \( k_{\text{cat}} \) refers to hydrolytic efficiency for inhibitors, as shown in Scheme VI.1) were determined as the ratio of inhibitor concentration to enzyme concentration necessary to decrease enzyme activity by 95% \( (19) \). The turnover numbers were determined after a 24 h incubation with increasing concentrations of the inhibitor. Incubations were done in a final volume of 300 µl and 25 µl of this reaction mixture were added to a 1 ml final volume to determine the residual enzyme activity using 100 µM nitrocefin.
Quantum Mechanical Calculations- Ab initio quantum mechanical calculations were performed on CWRU’s cluster facility to predict the Raman spectra of penems and model intermediate compounds using Gaussian 03 (156). Calculations were performed at the DFT level using the 6-31+G(d) basis set. DFT calculations were performed with Becke’s three parameters hybrid method using the correlation functional of Lee, Yang, and Parr (B3LYP) (187, 188). The vibrations giving rise to the most intense calculated peaks could be visualized using "GaussView", revealing which molecular vibrations contribute to the peaks.

Scheme VI. 1 Reaction scheme of β-lactamase-inhibitor interactions.

VI.3 RESULTS
VI.3.1 Kinetic data for penem 1 and 3 reacting with OXA-1 and OXA-24

Antibiotic susceptibility. To first determine whether penem inhibitors can be used as effective partners with clinical antibiotics, we performed microbiological assays to evaluate their ability to lower the MICs. To establish a comparison, we used piperacillin, a broad-spectrum penicillin family member, with the penems at a concentration of 4 µg/ml. We also used tazobactam at the same concentration as a comparator β-lactamase inhibitor. Tazobactam in combination with piperacillin became available in clinic in the
United States in 1993 and does extend piperacillin's activity against most class A β-lactamase producing strains. Against *E. coli* DH10B lacking OXA-1 or OXA-24 expression, the piperacillin MICs are 8 µg/ml, well within the susceptible range for piperacillin (CLSI guidelines) (189). In the bacterial strain where OXA-1 or OXA-24 is expressed, a high-level piperacillin resistance is observed (Table VI.2, piperacillin MIC is 512 µg/ml for OXA-1, 1024 µg/ml for OXA-24). When tazobactam was combined with piperacillin at the concentration of 4 µg/ml, we did not detect reduction in MICs for OXA-24 (1024 µg/ml, Table VI.2), and only slight reduction for OXA-1 (256 µg/ml, and no significant inhibition with piperacillin). This is consistent with the observation that the current clinically used β-lactamase inhibitors (tazobactam, sulbactam and clavulanate) are not effective against class D β-lactamases (39, 65, 183). Before measuring the inhibitory activity of penem inhibitors combined with piperacillin, we first tested whether penem 1 or penem 3 possess any intrinsic antibiotic activity against bacterial strains. The results showed the MICs for penem 1 or penem 3 alone are >1024 µg/ml, indicating that they alone do not bear any inhibitory activity.

Penems combined with piperacillin resulted in significant differences between OXA-1 and OXA-24. In OXA-1, a noticeable reduction in MICs by penem 1 or penem 3 was observed (512 to 8 µg/ml, both). However, in OXA-24, the MIC is not affected in the presence of penem 1 or penem 3, which shows that the two inhibitors are not effective against OXA-24 β-lactamase.
Table VI. 2 Minimum inhibitory concentration of Laboratory Isolates.

According to the Clinical Laboratory Standards Institute, MIC breakpoints for piperacillin and piperacillin/tazobactam are: ≤8 µg/ml, susceptible; 8-16 µg/ml, intermediate; ≥32 µg/ml, resistant.

<table>
<thead>
<tr>
<th>MICs (µg/ml)</th>
<th>Laboratory Isolate</th>
<th>Piperacillin</th>
<th>Piperacillin/ tazobactam*</th>
<th>Piperacillin/ penem 1</th>
<th>Piperacillin/ penem 3</th>
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<td><em>E. coli</em> blaoXA-24</td>
<td>1024</td>
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</tbody>
</table>

* The concentration of tazobactam, penem 1 and penem 3 is 4 µg/ml.

Kinetic parameters. In order to further demonstrate that OXA-1 and OXA-24 β-lactamases behave differently with penem inhibitors, we performed kinetic assays to observe the properties and activities of penems 1 and 3. Table VI.3 shows the $K_i$ and $IC_{50}$ of the penem compounds with the enzymes OXA-1 and OXA-24. The data suggest that penem 1 and penem 3 are good inhibitors against both OXA-1 and OXA-24 because their $K_i$ and $IC_{50}$ values are very low (at nM level). However, closer examination shows that the $IC_{50}$ value is much lower than $K_i$ in OXA-1 while higher in OXA-24. This suggests that, in OXA-24, the two penem inhibitors to some extent undergo subsequent hydrolysis before forming the stable acyl-enzyme complex. Thus, next we aimed to measure the turnover number for both enzymes. In Table VI.3, the results show that $t_n$ for OXA-24 is ~450 times higher than for OXA-1 (900 vs 2). Considering that the periplasmic
concentration of the OXA-10 β-lactamase in two clinical strains of *Pseudomonas* is about 4-15 µM (38), if OXA-1 or OXA-24 are at a similar concentration level as OXA-10, it would not be possible to inhibit OXA-24 under physiological conditions because of the high amounts of penems required. In summary, penem 1 and 3 are effective inhibitors for OXA-1 but not for OXA-24.

**Table VI. 3** Kinetic parameters of inhibition.

*K*₁ for the penems was determined by measuring initial steady-state velocities in the presence of a constant concentration of enzyme with increasing concentrations of inhibitors against nitrocefin (100 µM), the value was then corrected for nitrocefin affinity; IC₅₀ was determined by measurements of inhibitor concentration that reduces the initial velocities by 50% after 5 min pre-incubation of enzyme with inhibitor; *tₙ* was determined as the ratio of inhibitor concentration to enzyme concentration necessary to decrease enzyme activity by 95% after 24 h.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitors</th>
<th>OXA-1</th>
<th>OXA-24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>K</em>₁ (nM)</td>
<td>IC₅₀ (nM)</td>
<td><em>tₙ</em></td>
</tr>
<tr>
<td>Penem 1</td>
<td>50 ± 8</td>
<td>30 ± 8</td>
<td>2</td>
</tr>
<tr>
<td>Penem 3</td>
<td>380 ± 70</td>
<td>60 ± 5</td>
<td>2</td>
</tr>
</tbody>
</table>

VI.3.2 **Spectroscopic evidence for different reaction schemes in OXA-1 and OXA-24**

_Ultraviolet difference (UVD) spectroscopy: the role of carboxylated lysine._

Further evidence that OXA-1 and OXA-24 react differently with penem inhibitors is also obtained by UVD spectroscopy that has been widely used to provide insight into the nature of reactive intermediates or products formed during β-lactamase inactivation processes (22, 190, 191). We reacted penem 1 with OXA-1 or OXA-24 at different ratios
to see whether the reaction is stoichiometric. The data for penem 3 are not shown but they are similar to penem 1.

Figure VI.2 shows the reaction between penem 1 and OXA-1 or OXA-24 at 1: 1 or 1: 4 ratio (E: I). The peak at 280 nm represents the unreacted compounds and is assigned to an electronic transition located in the conjugated region involving the double bonds in the bicyclic ring and the methylenic double bond at C6 position, extending to the carbonyl group in the β-lactam ring. At 1: 1 ratio, all penem 1 molecules have been consumed because the 280 nm peak disappears, only leaving the product spectrum (Figure VI.2A). However, at 1: 4 ratio, almost three fourths of the penem 1 remained unreacted in OXA-1 (red line, Figure VI.2B) and, at this time, the enzyme is no longer active against nitrocefin (not shown). In OXA-24, the spectrum for 1: 4 ratio shows a similar pattern as the 1: 1 ratio that all penem 1s are hydrolyzed (blue line, Figure VI.2B). The enzyme is still active.

The unique peaks of the hydrolyzed-product spectra in OXA-1 (255 and 375 nm, Figure VI.2A) and OXA-24 (351 nm, Figure VI.2A) suggest the reaction undergoes different pathways. Based on the studies by Bethel et al. (183), we propose a reaction mechanism for penem 1 and OXA-1 (Scheme VI.2, modified from Bethel et al. (183)). Previous studies of compound BRL 42715 (Figure VI.1), a similar structure to penem 1, showed its reaction with a base, sodium methoxide in methanol, results in the formation of a seven-membered thiazepine with chromophores at 253 and 370 nm (108), which is consistent with the peaks in UVD spectroscopy of OXA-1. In addition, the NMR studies by Bethel et al. also support the conclusion that a similar intermediate, the 1,4-thiazepine derivative, is formed during the reaction between penem 1 and OXA-1 (183). Both these
serve as the experimental foundation for our proposed mechanism in Scheme VI.2 and support the existence of seven-membered thiazepine in the reaction between penem 1 and OXA-1.
Figure VI. 2 UV difference spectrum of OXA-1 and OXA-24 reacting with penem 1. The intense peak at 280 nm represents unreacted penem 1 (black line). The concentration of enzyme is 20 µM for OXA-1 and OXA-24. The concentration of inhibitor is 20 µM (1:1) or 80 µM (1:4). After the enzyme is incubated with penem 1 inhibitor, the spectrum is recorded every 30 s for 30 min. The spectra shown in the figure are the UV-difference spectra at 1 min, (the other spectra overlap together because the reaction occurs rapidly in solution). (A) OXA-1 or OXA-24 reacting with penem 1 at 1:1 ratio (E: I); (B) OXA-1 or OXA-24 with penem 1 at 1:4 ratio.
For OXA-24, in terms of the turnover number (900 for penem 1), the situation mostly mimics the reaction pathway for penicillin hydrolyzed by OXA-24 β-lactamase (39). Under normal conditions, penem inhibitors are treated as substrates of the enzyme. Thus, we propose another mechanism for penem 1 and OXA-24 (Scheme VI.3, adapted from the mechanism for penicillin hydrolysis (39)). In contrast to the reaction in OXA-1, the carboxylated Lys84 in the active site of OXA-24 utilizes a catalytic water molecule to deacylate the Ser81. As a result, the penem 1 inhibitor is hydrolyzed, the enzyme is regenerated since Lys84 is not decarboxylated (as discussed below) and hydrolyzes the next arriving inhibitor molecule.

In order to confirm the role of carboxylation and decarboxylation of the active-site lysine, we undertook assays with nitrocefin (NCF) and bicarbonate. The nitrocefin assay shows that OXA-24 can be inhibited by a high concentration of penem 1 (E: I = 1: 2000), although the addition of 100 mM NaHCO₃ can reactivate the enzyme because nitrocefin is hydrolyzed again (Figure VI.3A). A recent study in our laboratory shows that one penam sulfone inhibitor, SA-1-204 (Figure VI.1), can effectively inhibit OXA-24 by decarboxylating the Lys84 in the active site, yet the enzyme is recarboxylated and becomes active again after adding 100 mM NaHCO₃ as a source of CO₂ molecules in the solution (65). These findings together indicate that, in OXA-24, a high concentration of penem 1 causes the decarboxylation of Lys84, which has been shown to be critical for the enzyme's deacylation (65, 176).

In contrast, the nitrocefin assay shows that a low concentration of penem 1 effectively inhibits OXA-1, but nitrocefin does not react in mixture of OXA-1 and penem 1 (E: I = 1: 4) after treatment with 100 mM NaHCO₃ (Figure VI.3B). Considering that
OXA-1 and OXA-24 belong to class D β-lactamases that use a carboxylated lysine side chain (Lys70 and Lys84, respectively) to aid catalysis, we hypothesize that penem 1 can also cause the essentially irreversible decarboxylation of OXA-1.
Figure VI. 3 Recarboxylation of the lysine can restore the enzyme activity in OXA-24, but not in OXA-1. A, black line: 1 µM OXA-24 was incubated with 100 µM NCF in HEPES buffer (10 mM, pH 7.5); red line: 1 µM OXA-24 was first incubated with 2 mM penem 1, then 100 µM NCF was added; blue line: Reagents were added in the order of 1 µM OXA-24, 2 mM penem 1, 100 µM NCF and 100 mM NaHCO₃. B, black line: 1 µM OXA-1 was incubated with 100 µM NCF in HEPES buffer (10 mM, pH 7.5); red line: 1 µM OXA-1 was first incubated with 4 µM penem 1, then 100 µM NCF was added; blue line: Reagents were added in the order of 1 µM OXA-1, 4 µM penem 1, 100 µM NCF and 100 mM NaHCO₃.

Raman studies of penem 1-OXA-1/OXA-24 reactions in solution. Using the rapid mix-rapid freeze protocol developed in our laboratory (54), we examined the reaction
mixture between OXA-1 or OXA-24 with penem 1, 1 second after mixing. A ratio of 1: 2 (E: I) was used, we expect for OXA-1 to see the inhibited complex ($t_n$ is 2, Table VI.3), but for OXA-24 to see predominantly hydrolyzed product since $t_n$ is about 900 (Table VI.3). These predictions are supported by the UVD spectra discussed in the previous section.

Figure VI.4 compares the Raman spectrum of free penem 1 in aqueous solution with the spectrum of the freeze-dried reaction mixtures obtained 1 second after mixing and flash freezing. We first employed *ab initio* quantum mechanical calculations to help interpret the spectrum of the substrate (upper trace, Figure VI.4), which serves as a basis for the following analysis. Based on Gaussian calculations, the peak at 1687 cm$^{-1}$ in the spectrum of unreacted penem 1 is assigned to the methylenic double bond coupled to the carbonyl group in the β-lactam ring (Table VI.4), which is expected to change markedly when the β-lactam ring opens or the hybridization at C6 changes. Another characteristic feature is at 1757 cm$^{-1}$, due to the carbonyl group (C=O) in the intact lactam ring. The disappearance of this peak suggests the opening of the lactam ring due to acylation of the enzyme (53).

For OXA-1 reacting with penem 1, we see evidence for two species. Quantum mechanical calculations indicate that the band at 1630 cm$^{-1}$ is from species 4 in Scheme VI.2 and is due essentially to the methylenic double bond stretch coupled to the ester bond (-O-C(=O)-C=C-) in acyl-enzymes. (Table VI.4), although this mode is partially delocalized over adjacent conjugated bonds. Chemical structures including this acrylic group (R$_1$-O-C(=O)-C=C-R$_2$) all give rise to intense Raman peak around 1628 cm$^{-1}$ (data
This assignment is strengthened by the observation that a similar mode occurs at 1645 cm$^{-1}$ for the reaction with OXA-24 (Figure VI.4).

**Figure VI. 4** Raman difference of spectra of reactions between penem 1 and OXA-1 or OXA-24 in solution. Unreacted penem 1 spectrum (upper trace, 10 mM, in H$_2$O). Enzyme (86 µM) and inhibitor (172 µM) were incubated at the ratio of 1: 2 (E: I). The reactions were quenched by liquid nitrogen after 1 s. The ice was then freeze-dried and characterized by Raman microscopy. 4(2), 9(2): species 4 and 9 in Scheme VI.2; C(3), F(3): species C and F in Scheme VI.3; C5-S1: C-S bond of the thiazole ring attached to the β-lactam ring.

The latter mode is due to hydrolyzed species E and/or F in Scheme VI.3 (see below). The Carey group reported in the 1980s that the ethylenic stretch in α, β conjugated molecules, such as cinnamic acid and furylacryloyl acid, increases by 10-15 cm$^{-1}$ upon ionization of the acid group (see Table II in (192)). Species E and F (Scheme VI.3) are the ionized form and species 4 (Scheme VI.2) is the neutral ester form,
respectively. Important evidence for the second species comes from the broad unresolved band around 1500 cm^{-1} (Figure VI.4). The quantum mechanical calculations for the seven-membered ring seen in Scheme VI.2 show two intense features near 1510 and 1492 cm^{-1} (Table VI.4). These features arise from the double bonds in the seven-membered ring and are not resolved in Figure VI.4. Meanwhile, we do not detect a mode characterizing an ester near 1725 cm^{-1}. This argues that we are detecting the species with the ionized -COO\(^-\) (species 9) and not the ester-like acyl enzyme (species 6). However, we cannot definitively argue in favor of species 9 since in some instances the Raman spectrum of acrylic acid ester, the C=O feature has low intensity. Interestingly, a characteristic C-S stretch is also present at 715 cm^{-1}, giving additional support to the formation of the seven-membered ring. It is noteworthy that Ke et al. have detected the same seven-membered ring species in the X-ray structure of SHV-1 class A β-lactamase complexed with penem 1 (193).

The reason we have evidence for two species from the OXA-1 reaction is that with a \( t_n \) of 2, we expect to produce a population of product species 9 in Scheme VI.2 and a population of covalently inhibited stable acyl-enzyme, species 4 in reaction scheme VI.2. This analysis predicts that Lys70 is decarboxylated after the first cycle and thus, in the second cycle, species 4 is bound irreversibly. An underlying hypothesis is that acylation can occur without the participation of Lys70 but that this residue is essential for deacylation.

The solution data for OXA-24 and penem 1 are expected to be very different since the \( t_n \) is high for OXA-24 (900, Table VI.3), we should see a lot of penem 1 transformed into hydrolyzed product. This expectation is confirmed by the Gaussian calculations. The
latter show that species F (Scheme VI.3) has an intense Raman band near 1645 cm$^{-1}$ as seen in Figure VI.4. This is due to the C=C stretch in molecule F where $-\text{COO}^-$ shifts new C=C 10-15 cm$^{-1}$ to higher wavenumber than in the neutral molecule C, as discussed above and listed in Table VI.4. The calculations also reproduce the intense Raman band near 716 cm$^{-1}$ from the thiazole five-membered ring of species F (Scheme VI.3) and support the presence of the thiazole ring.
Scheme VI. 2 Proposed mechanism for penem 1 and OXA-1.
Scheme VI. 3 Proposed mechanism for penem 1 and OXA-24.
Table VI. 4 Raman peak assignments for the major peaks in penem 1 complexed with OXA-1 or OXA-24 difference spectra shown in Figure VI.4.

The structures of the proposed intermediates in the reaction pathway were sent to the HPCC at CWRU for calculating the theoretical Raman spectrum.

<table>
<thead>
<tr>
<th>Species</th>
<th>Observed Raman peaks (cm$^{-1}$)</th>
<th>Calculated Raman peaks (cm$^{-1}$)</th>
<th>Calculated relative Raman intensities</th>
<th>Peak assignments</th>
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</thead>
<tbody>
<tr>
<td>OXA-1$^a$</td>
<td>1757</td>
<td>1768</td>
<td>13</td>
<td>C=O of β-lactam ring</td>
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<tr>
<td></td>
<td>1687</td>
<td>1680</td>
<td>342</td>
<td>methylenic double bond at C6</td>
</tr>
<tr>
<td></td>
<td>1463</td>
<td>1476</td>
<td>105</td>
<td>coupled to C=O of β-lactam ring</td>
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<tr>
<td></td>
<td>1448</td>
<td>1450</td>
<td>73</td>
<td>C=O of thiazole ring</td>
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<td></td>
<td>720</td>
<td>717</td>
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<td>-N=N=C- of bicyclic ring</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>-C=S between S1 and C5</td>
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<td>1630</td>
<td>1625</td>
<td>152</td>
<td>methylenic double bond at C6</td>
</tr>
<tr>
<td></td>
<td>---</td>
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<td>C=O of thiazole ring</td>
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<td>46</td>
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<td>-C=S between S1 and C5</td>
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<td>41</td>
<td>-N=N=C- of bicyclic ring</td>
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<td>715</td>
<td>721</td>
<td>2</td>
<td>-C=S of thiazepine ring</td>
</tr>
</tbody>
</table>

$^a$ The values in this column represent the peaks from the spectrum of OXA-1/OXA-24 reaction with penem 1 in Figure VI.4.

Raman Analysis of penem 1 reaction with OXA-1/OXA-24 single crystals.

Whereas the solution studies had enzyme: inhibitor ratio of 1: 2, the crystals suspended in
a hanging drop containing inhibitor have access to a huge excess of inhibitors - 10 mM in a 5 µl drop. For OXA-1 and penem 1, it is again likely that the enzyme goes through a maximum of 2 cycles.

Figure VI.5A shows the Raman difference spectra of penem 1 reaction in OXA-1 single crystal (underneath the spectrum of unreacted penem 1 in aqueous solution). The results show that the reaction occurs slowly because there is no intense Raman signal at 3 min (Figure VI.5A), indicating that penem 1 molecule has not entered the crystal and the active site of OXA-1 by the time. At 15 min, some new peaks appear, e.g. at 1656 cm$^{-1}$. At 30 min, another peak at 1628 cm$^{-1}$ intensifies and remains stable up to 1 h, this is assigned below. The intense at 1656 cm$^{-1}$ is assigned to the protonated imine (-C=NH$^+$-) of species 5 (Scheme VI.2). This protonated imine due to the opening of the five-membered ring is a common intermediate in sulbactam, tazobactam and clavulanate reaction with SHV-1 β-lactamase, which also give rise to intense peak at 1656 cm$^{-1}$ (61).

On the basis of the calculations discussed above, we predict the seven-member ring product (species 9, Scheme VI.2) will generate two intense peaks around 1490 and 1500 cm$^{-1}$. These were detected in solution (above) but they are not obvious in Figure VI.5A. The reason may be due to the low-abundance or fast-release. Considering that there are still intense substrate peaks together with the intermediate peaks under soak in conditions, in order to see what species is finally left in the active site of OXA-1, we performed a soak out experiment. By immersing the reacted crystal in holding solution containing no substrate we remove non-covalently bound substrates or products, leaving the covalently bound species in the active site. After 1 h incubation with penem 1, we transferred the crystal to a new hanging drop without any inhibitor molecule and took the spectrum of
the crystal. In the soak-out experiment, the peaks at 1761, 1689 and 1654 cm\(^{-1}\) disappear, only the 1629 cm\(^{-1}\) peak remains, strongly suggesting that the species at 1629 cm\(^{-1}\) represents an entity covalently bound in the active site (Figure VI.5A).
Figure VI. 5 Raman difference spectra of OXA-1 or OXA-24 single crystals reaction with penem 1. (A) Raman difference spectrum of OXA-1 and penem 1 at 3, 15, 30 min in the presence of PEG 8000. Control spectrum of penem 1 (upper trace) was first recorded in the presence of PEG. After penem 1 was soaked in, the spectra were taken at above indicated time points. (B) Raman difference spectrum of OXA-24 and penem 1 at 4, 15, 30 min in the presence of PEG 2000. Control spectrum of penem 1 (upper trace) was first recorded in the presence of PEG. After penem 1 was soaked in, the spectra were taken at above indicated time points.
In accord with the solution studies (above), the 1629 cm\(^{-1}\) feature is assigned to the stable acyl enzyme, species 4 in Scheme VI.2, that resists hydrolysis because Lys70 is decarboxylated in the first cycle of the reaction. Thus, it appears that species 4 in reaction Scheme VI.2 is the final stable product in the crystal after 30 min soak in and 30 min soak out.

In the crystal reaction for OXA-24, we predict that reaction cycle will occur many times, since the \(t_n\) for penem 1 is \(\approx 900\) (Table VI.3). Figure VI.5B shows the reaction between penem 1 and OXA-24 single crystals. As for OXA-1, penem 1 enters the active site slowly because at 4 min, there are no intense substrate or intermediate peaks. At 15 min, a broad peak at 1628 cm\(^{-1}\) appears and another intense peak at 1647 cm\(^{-1}\) is present at 30 min. As described above, based on the quantum mechanical calculations, the peak at 1647 cm\(^{-1}\) is due to the released species F (Scheme VI.3), which is produced in large amounts due to multiple reaction cycles. Again, in order to remove the unreacted substrate and detect the covalently-bound species in the active site, the soak out experiment was conducted. Interestingly, we saw the 1628 cm\(^{-1}\) peak, the same peak as in the active site of OXA-1. This indicates that, after penem 1 reacts with OXA-1 or OXA-24, they both have the same species finally remaining, covalently bound, in the active site. We observe the 1628 cm\(^{-1}\) band due to species C in Scheme VI.3 because in OXA-24 Lys84 has finally decarboxylated after about 900 cycles, and acyl-enzyme species C can no longer be hydrolyzed. The same hypothesis applies as for OXA-1, acylation is still possible without a carboxylated lysine, but deacylation cannot occur, or occurs very slowly, at best.
VI.3 DISCUSSION

The results discussed above indicate that OXA-1 and OXA-24 react differently with the penem inhibitors. The deacylating lysine in both enzymes can be decarboxylated by penem 1; OXA-1 resists recarboxylation and thus cannot hydrolyze the acyl-enzyme. An unusual finding in our work is that, at modest conditions, penem 1 and 3 appear to be ineffective against OXA-24. Based on the kinetic data, and the reactions in crystal and in solution, we propose that penem 1 causes decarboxylation of Lys70 in the active site of OXA-1 in the first cycle rendering the enzyme inactive. The next arriving molecule forms the acyl-enzyme complex (species 4, Scheme VI.2), where carboxylation of Lys70 is not essential for acylation (176, 194), but does not react further because the enzyme loses the deacylation function following decarboxylation of Lys70. However, decarboxylation of Lys84 in the active site of OXA-24 occurs only under harsh condition. The enzyme remains active and hydrolyzes penem 1 again and again. The difference in the nitrocefin assay indicates that OXA-1 and OXA-24 differ in both decarboxylation and recarboxylation steps. OXA-24 not only decarboxylates with difficulty but also recarboxylates with ease. In contrast, OXA-1 decarboxylates easily but recarboxylates with difficulty. We now discuss evidence from the literature to explain the difference between OXA-1 and OXA-24.

Previous studies by the Mobashery group showed that the BlaR1 sensor, a signal transducer found in Staphylococcus aureus bacterium, has a serine and carboxylated lysine motif in the active site (147, 195). Its protein sequence and overall folding indicate that it is evolutionarily related to class D β-lactamases. When the sensor reacts with antibiotics, it forms an acyl-enzyme complex and the Lys62 undergoes decarboxylation,
switching the receptor to the "on" state and continuously inducing the expression of the β-lactamase (146-149). In order to find out why BlaR1 sensor decarboxylates easily compared to OXA enzymes, Birck et al., in a related study (147), used the X-ray coordinates of OXA-10 as a model to calculate the energy barrier to decarboxylation. Before the decarboxylation occurs, the carboxylated lysine is protonated following active-site serine acylation (143, 147, 196). Based on their calculations on OXA-10 (Figure VI.6), if the protonation occurs on the oxygen in the -NH-COO of the side chain, there is a huge barrier (~40 kcal/mol) for the decarboxylation of Lys70. However, if the protonation is on the ζ-nitrogen in the NH of the side chain, there is no barrier for the decarboxylation. Thus in BlaR1 sensor, if ζ-N protonation can be prevented, Lys62 will remain carboxylated and the acyl-enzyme can be hydrolyzed. Based on this consideration, Birck et al. changed the Lys62 in BlaR1 to S-(4-butanoate)-cysteine by chemical mutagenesis (197) to see whether it is sufficient to convert the BlaR1 sensor from a susceptible receptor to an antibiotic-resistant enzyme (143). The results show that the cysteine derivative does not undergo decarboxylation and the variant hydrolyzes the acyl-enzymes formed from a broad spectrum of antibiotics (143). Kinetic studies indicate that this behavior is reproduced in OXA-10 (38). Returning to the OXA-1 and OXA-24 systems, we postulate that, in OXA-1, it is the ζ-nitrogen of Lys70 that undergoes protonation in the deacylation process and this brings about decarboxylation (Scheme VI.2, species 7,8,9). Even though the Ser67 is free now and can react with the next penem 1 molecule, forming the acyl-enzyme complex, the Lys70 is inactive and loses the deacylation function because of the decarboxylation. Previous studies have also shown that BlaR1 sensor shares more similar properties with OXA-1 than OXA-24 (147, 153,
In OXA-24, we postulate that it is the COO’ that undergoes protonation (Scheme VI.3, species D), thus the enzyme is still active and can hydrolyze the next penem 1 molecule.

Figure VI. 6 QM/MM calculations using the X-ray coordinates of the OXA-10 active site reveal the protonation of the ζ-nitrogen leads to a barrierless decarboxylation of the lysine carbamate, (modified from Birck et al. ref 147).

Regarding the recarboxylation process, we found that OXA-24 can be reactivated by the addition of 100 mM NaHCO₃, but OXA-1 cannot. According to the calculation results by Schlegel and Mobashery’s groups on OXA-10 β-lactamase (150), the barrier for unassisted carboxylation of neutral lysine is as high as 30 kcal/mol. However, if the recarboxylation is assisted by a water molecule close enough (2.4 ~ 2.8 Å) to the lysine in the active site, the energy barrier is much lower (exothermic with a barrier of 14 kcal/mol). This led us to examine the active sites of both enzymes to determine if there is a water molecule that exists near the lysine in the active site of OXA-24 but not in OXA-1. Figure VI.7 shows the positions of the water molecules surrounding the lysine residue in the active of OXA-1 and OXA-24. In OXA-1, the closest water molecule is 4.6 Å away from Lys70; in OXA-24, it is 2.6 Å. Thus, there is a water molecule in OXA-24 that can be utilized to catalyze the recarboxylation. This water molecule, together with Trp167 can form H-bonds with CO₂ group (2.6 and 2.9 Å, respectively), positioning the
CO₂ in the right place ready for the attack from ζ-nitrogen in Lys84 to form the carboxylated lysine seen in Figure VI.7. A detailed model for the reaction scheme during lysine carboxylation is shown in Li et al. (150). The X-ray structure of OXA-24 complexed with an inhibitor LN-1-255 also indicates that the bulky side group in the latter seems to force out the water molecule, leading to deacylation deficiency (39). Considering that the CO₂ concentration is about 1.3 mM in cells (169), OXA-24 has easy access to the CO₂ group and becomes fully carboxylated. This may explain, in part, why clinical strains harboring blaOXA-24 genes have multiple-drug resistance to β-lactams.
Figure VI. 7 The water position in the active site of OXA-1 (A) and OXA-24 (B). Water molecule is shown in red sphere. Selected interacting residues are labeled, and hydrogen bonds are indicated by dashed lines. The PDB entry: OXA-1 (1M6K), OXA-24 (3G4P).

VI.4 CONCLUSIONS

In conclusion, the two variants of class D β-lactamase OXA-1 and OXA-24 are found to react differently with 6-methyledene penems. This is ascribed to the differential
stability of the carboxylated lysine in the active site. The catalytic difference between OXA-1 and OXA-24 also provides us new insight into inactivation mechanism by OXA carbapenemases. Compared to OXA-1, OXA-24 is not only difficult to decarboxylate but also can easily be recarboxylated. This may be also consistent with their relative pathogenic effects as the pathogen harboring blaOXA-24 (A. baumannii) has been more problematic in the clinic. Based on these findings, a novel inhibitor design of class D β-lactamase should aim to accelerating the decarboxylation step and also importantly, retarding the recarboxylation step, unlike the traditional strategy in class A and C β-lactamases of blocking Glu166 (or its homologue) assisted hydrolysis.
Appendix

Chapter III: Detecting a Quasi Stable Imine Species on the Reaction Pathway of SHV-1 β-Lactamase and 6β-(Hydroxymethyl)penicillanic Acid Sulfone

A  Sulbactam in $D_2O$
Solvent: $D_2O$
600 MHz

B  Sulbactam in NaOH - 10 min
Solvent: $D_2O$
600 MHz
**Figure III.S 1** Following base hydrolysis of sulbactam by $^1$H NMR. 50 μl 5 mg/ml sulbactam (in D$_2$O) was incubated with 50 μl 1 M NaOH (in D$_2$O). The sample was applied to NMR at different time points.

Figure III.S1A shows the 600 MHz NMR spectrum of sulbactam in D$_2$O. Using assignments given by BioChemDraw, the doublet near 1.4 ppm is due to the two CH$_3$ groups, the peaks 3.2 and 3.4 ppm are due to the 2Hs on C6 and the two peaks at 4.3 and 4.9 ppm are due to Hs on C5 and C3 (or vice versa). The feature at 4.6 ppm is due to HOD.

At pD = 14 after 10 minutes (Figure III.S1B), the peaks near 0.8 ppm are due to the 2CH$_3$ Hs, the doublet near 1.3 ppm is due to a trace of unreacted sulbactam, the peak at 3.2 ppm is due to the H on C3 of the enamine, the peak near 3.4 is from the CH$_2$ group of the imine, at 4.4 ppm from the H on C3 of the imine, the 7.05 ppm from the H on C5 of the imine, and the peak at 8.0 ppm from the two Hs on -CH=CH- of the enamine.

At 2 hours at pD 14 (Figure III.S1C) the relative intensities of the peaks from enamine have grown compared to those of the imine. Thus, the NMR is recording the imine to enamine tautomerization with a similar time dependence as seen in the Raman experiments in Figure III.2A.
Figure III.S 2 Reaction of 6-D,D-sulbactam with SHV-1 displays similar patterns as sulbactam, but the transition from imine to enamine is slower than in sulbactam due to the isotope effect.
Chapter V: Carboxylation and Decarboxylation of Active Site Lys 84 Controls the Activity of OXA-24 β-Lactamase of Acinetobacter baumannii: Raman Crystallographic and Solution Evidence

Supplemental Text

1. Acquisition of Raman difference spectrum

   For the spectrum of unreacted inhibitor SA-1-204, a buffer spectrum (W/O 28% PEG 2000) was obtained in a 5 µL hanging drop with a power of 80 mW (Spectrum 1). Next, spectrum of 5 mM inhibitor SA-1-204 was obtained in the same buffer as the above control, respectively (W/O 28% PEG 2000) (Spectrum 2). The Raman difference spectrum of inhibitor SA-1-204 was obtained by subtracting Spectrum 1 from Spectrum 2. Spectrum subtraction was performed using GRAMS/32 software (Galactic Industries, Inc.).

   For the spectrum of bound inhibitor SA-1-204 in the active site of OXA-24, a spectrum of OXA-24 crystal in holding solutions containing 0.1 M HEPES (pH 7.5), 0.1M sodium acetate and 28% PEG 2000 was firstly recorded (Spectrum 3). Then, a spectrum of its mother liquor, obtained by shifting the hanging drop ~50 µm so that the laser focal point is away from the crystal (Spectrum 4). After Spectrum 4 was subtracted from Spectrum 3, the Raman difference spectrum of OXA-24 crystal was obtained (Spectrum 5). After inhibitor SA-1-204 was soaked in (final concentration is 5 mM), the time-dependent spectrum was recorded and the laser focal point on the crystal was at the same point as that before soak in. The spectrum of crystal after soak in (Spectrum 6) and new buffer (Spectrum 7) were obtained, respectively. A similar spectral subtraction was
performed to give Raman difference spectrum of crystal after soak in (Spectrum 8).

Finally, a Raman spectrum of bound SA-1-204 in the OXA-24 crystal plus any conformational changes of the enzyme was obtained by subtracting the following two spectra:

Raman difference spectrum of bound ligand = Spectrum 8 - Spectrum 5
= [(Spectrum 6 + Spectrum 7) - (Spectrum 7)] - [(Spectrum 3 + Spectrum 4) - Spectrum 4]

In this final trace, the intensities of the Raman peaks were ratioed against 1000 cm\(^{-1}\) phenyl ring mode to enable us to compare intensities among spectra. The intensities of the modes in Figure V. 2 are about 3% compared to the intense peaks in the acyl-enzyme mother spectrum.

2). Concentration of active sites in a single crystal is calculated as follows:

\[
\frac{\text{number of asymmetric subunits per unit cell}}{\text{volume per unit cell}} \times (6.02 \times 10^{23} \text{ Mole}^{-1})
\]

For OXA-24 crystal, it belongs to space group \(P4_12_12\). There are 8 asymmetric subunits in a unit cell. The volume per unit cell is 102 \(\times\) 102 \(\times\) 86 \(\text{Å}^3\). A value of \(\approx\)15 mM is obtained for the concentration of OXA-24 active sites in a single crystal.
Figure V.S 1 Overall difference Raman spectra of OXA-24 β-lactamase complexed with SA-1-204 for selected time points.
Figure V.2 Overall kinetic depiction of the main peaks intensities over time. A. Kinetic depiction of the substrate peaks intensities at 1695 cm\(^{-1}\), 1588 cm\(^{-1}\), 1570 cm\(^{-1}\), 1216 cm\(^{-1}\) over time. B. Kinetic depiction of the intermediate peaks intensities at 1659 cm\(^{-1}\), 1550 cm\(^{-1}\), 1447 cm\(^{-1}\), 1322 cm\(^{-1}\) over time.
Figure V.S 3 Difference absorbance spectrum of SA-1-204 in OXA-24 solution with 10 mM HEPES (pH 7.5). The spectrum of OXA-24 and SA-1-204 were first acquired separately. Once they were mixed, spectrum was acquired at the intervals of 30 seconds in a 30 minutes time range. A. The reaction undergoes one cycle in the absence of NaHCO₃. 20 µM OXA-24 and 60 µM SA-1-204 were mixed. Without NaHCO₃, the spectrum from 1 minutes to 15 minutes are from the mixture of product and unreacted substrates. B. The reaction undergoes more than one cycle in the presence of 100 mM NaHCO₃. With NaHCO₃ in solution, the reaction happens quickly and all the substrates (black line) are transformed into product (from 1 minute to 15 minutes).
Bibliography


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