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"Mind the Gap": Raman Evidence for Rapid Inactivation of CTX-M-9 β-Lactamase Using Mechanism-Based Inhibitors that Bridge the Active Site

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Supporting Information

ABSTRACT: CTX-M β-lactamases are one of the fastest growing extended-spectrum β-lactamase (ESBL) families found in *Escherichia coli* rendering this organism extremely difficult to treat with β-lactam antibiotics. Although they are grouped in class A β-lactamases, the CTX-M family possesses low sequence identity with other enzymes. In addition, they have high hydrolytic activity against oxyimino-cephalosporins, despite having smaller active sites compared to other ESBLs in class A. Similar to most class A enzymes, most of the CTX-M β-lactamases can be inhibited by the clinical inhibitors (clavulanic acid, sulbactam, and tazobactam), but the prevalence of inhibitor resistance is an emerging clinical threat. Thus, the mechanistic details of inhibition pathways are needed for new inhibitor development. Here, we use Raman microscopy to study the CTX-M-9 inactivation reaction with the three commercially available inhibitors and compare these findings to the analysis of the S130G variant. Characterization of the reactions in CTX-M-9 single crystals and solution show the formation of a unique cross-linked species, probably involving Ser70 and Ser130, with subsequent hydrolysis leading to an acrylate species linked to Ser130. In solution, a major population of this species is seen at 25 ms after mixing. Support for this finding comes from the CTX-M-9 S130G variant that reacts with clavulanic acid, sulbactam, and tazobactam in solution, but lacks the characteristic spectroscopic signature for the Ser130-linked species. Understanding the mechanism of inactivation of this clinically important ESBL-type class A lactamase permits us to approach the challenge of inhibitor resistance using knowledge of the bridging species in the inactivation pathway.

Despite their discovery nearly 70 years ago, β-lactam compounds remain the most commonly used and clinically important antibiotics to combat bacterial infections. Regrettably, the production of β-lactamases (E.C. 3.5.2.6) is the most widespread cause of resistance to β-lactam antibiotics in Gram-negative bacteria, nullifying their clinical impact. Four major groups of β-lactamases are present in prokaryotic organisms (Classes A–D). Classes A, C, and D use an active site serine to hydrolyze the β-lactam, while class B enzymes use an active site Zn.[1,2]

CTX-M β-lactamases are some of the most prevalent extended spectrum β-lactamases (ESBLs) found in *Escherichia coli* and are able to hydrolyze oximino-cephalosporins, such as cefotaxime. As class A β-lactamases, CTX-Ms share less than 40% identity with TEM- and SHV-type enzymes, whereas they share more than 90% identity among themselves and form a more homogeneous ESBL family.[3−5] Although CTX-M-9 has a smaller active site compared to SHV and TEM enzymes, this β-lactamase efficiently binds and hydrolyzes large cephalosporin molecules,[4−6] which were designed with bulky C7 side chains to make them inherently less susceptible to β-lactamase activity.[7,8]

One of the most successful approaches to overcome the destructive action of the β-lactamase enzymes is to use a β-lactamase inhibitor, in synergy with β-lactam antibiotics, to inactivate the β-lactamase enzyme.[2] Clavulanic acid, sulbactam, and tazobactam, are three well-known β-lactamase inhibitors that have been used clinically for more than three decades. However, the advent of inhibitor resistant β-lactamase enzymes underscores the importance of developing novel β-lactamase inhibitors. To design effective β-lactamase inhibitors that meet the challenges of new enzymes and inhibitor resistant variants, the details of the inactivation pathways for wild type (wt) and mutant enzymes must be understood.[2,8]

Scheme 1 shows the outline of the proposed mechanism of inactivation for a class A enzyme by tazobactam, a pathway known to be similar for the other two clinical inhibitors. This reaction scheme is well established for SHV and TEM enzymes based on numerous studies.[9−14] In the first step, the active-site serine 70 attacks the carbonyl carbon of the β-lactam ring and forms an acyl-enzyme (see Scheme 1), followed by opening of the five-membered ring. Serine 130 has been invoked as the catalytic acid in protonation of the lactam nitrogen leaving group.[15] The acyl group then can be removed from the active site by hydrolysis. Alternatively, the imine acyl-enzyme can rearrange to produce longer-lived enamine-like species that inhibit the

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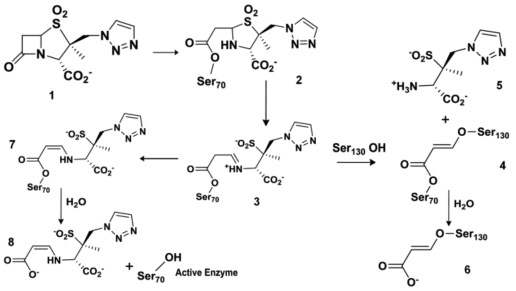
Scheme 1. Simplified Reaction Pathway of a Class A β-Lactamase for Tazobactam



enzyme. Less prevalent is attack on the imine by a second serine (Ser130, which is ~3.1 Å from Ser70[7]) side chain to form a covalently cross-linked unreactive enzyme.

Given the major differences in amino acid sequences between the CTX-M family and the other common class A β-lactamases such as TEM and SHV, the detailed mechanism of inhibition requires investigation.[8] We hypothesized that the inactivation pathway might be different compared to that for other class A enzymes. Consequently, Raman spectroscopy is used here to study the reaction between CTX-M-9 β-lactamase and the three inhibitors, clavulanic acid, sulbactam, and tazobactam *in crystallo*, and in solution in real time. A major advantage for Raman studies is that they can obtain millisecond time-resolved data on intermediates in dilute aqueous solution to compare with intermediates in single crystal. In turn, these data enable us to interpret Raman data for the corresponding reaction occurring in bacterial cells *in vivo*.[16]

Using a single crystal of CTX-M-9 in a hanging drop and Raman difference microscopy,[17,18] the reactions of CTX-M-9 enzyme with each inhibitor were studied separately. Figure 1 shows the Raman difference spectra of wild type CTX-M-9 single crystal with clavulanic acid, tazobactam, and sulbactam after 25 min of inhibitor soak-in. The basis for interpreting the Raman peaks seen for the three inhibitors between 1600 and 1700 cm^{-1} stems from our extensive studies of wt SHV-1 and SHV-1 E166A β-lactamases.[9,13,14,19−23] In summary, the peaks near 1603 cm^{-1} are due to enamine species (Scheme 1, species 7). Peaks in the 1650−1670 cm^{-1} region are due to the presence of pro-imines.[9,21] An important observation is the intense mode, seen for all three inhibitors, near 1520 cm^{-1}. This feature was only observed once in our many earlier studies. Totir et al. reported a weak peak near 1530 cm^{-1} when sulbactam was soaked into E166A SHV-1 β-lactamase at 10−29 h soaking time; a peak was not seen at 75 min with wt β-lactamase.[22] Extensive studies using dideuterated sulbactam supported by quantum mechanical calculations confirmed that the 1530 cm^{-1} feature could be assigned to the mode from the hydrolysis product (Scheme 1, species 6) of two serine residues covalently cross-linked by an acrylic-like fragment derived from the inhibitors. Although cross-linking has been suggested to occur in other β-lactamase−inhibitor reactions,[24,25] the present study is the first time that cross-linking has been shown to occur within 25 ms in an aqueous solution reaction.

A remarkable property of the 1520−1525 cm^{-1} peak in the three spectra seen in Figure 1 is that it is intense at 25 min and is seen with slightly lesser intensity at shorter soak-in time (see Supporting Information Figures S1 and S2). This compares with the several hours needed to observe the corresponding peak in crystal of SHV-1 E166A and sulbactam.[22]

The novel feature of the crystal data is underscored by the appearance of the 1520 cm^{-1} peak in aqueous solutions following mixing in a rapid mix−rapid freeze system. (The experimental protocol for this experiment is given in ref 21.) Figure 2 presents the Raman difference spectra of CTX-M-9 reacting with clavulanic acid in solution and frozen after 25 ms, 1 s, and 5 s and subsequently trapped in freeze-dried powder. A substantial population of β-alkoxyacrylate (Scheme 1, species 6) is present within 25 ms, and this population continues to grow at the expense of the enamine and imine species, 1 s and 5 s after mixing. A similar set of reactions was repeated for CTX-M-9 with sulbactam or tazobactam using the rapid mix−rapid freeze technique. Figure 3 shows the Raman difference spectra of CTX-M-9 reacted with sulbactam and tazobactam after 5 s.

Two major Raman features around 1510 and 1600 cm^{-1} in both spectra are assigned to β-alkoxyacrylate and enamine species, respectively. Comparison of Raman spectra of sulbactam and tazobactam after 5 s of the reaction (Figure 3) with earlier

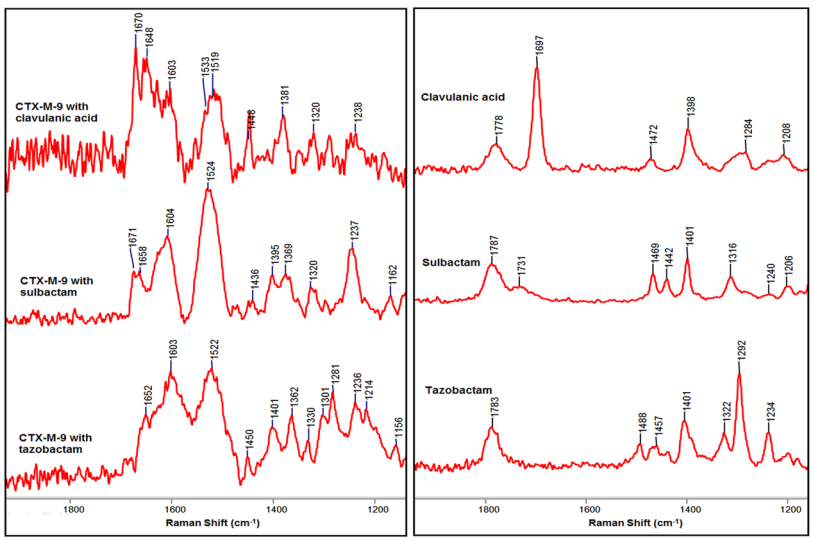


Figure 1. Raman difference spectra of CTX-M-9 wild type single crystal with clavulanic acid, sulbactam, or tazobactam after 25 min soak-in (left) and Raman spectra of clavulanic acid, sulbactam, and tazobactam desolved in water (right).

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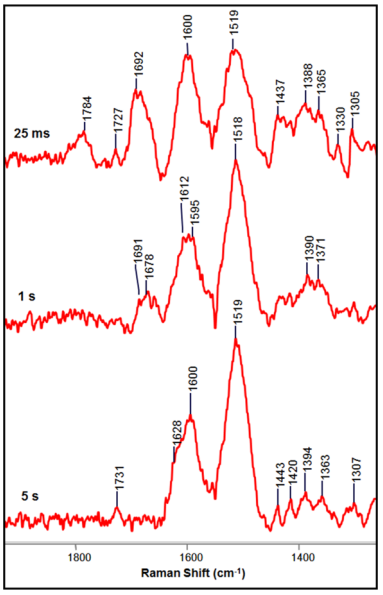

Figure 2. Raman difference spectra of CTX-M-9 with clavulanic acid (1 to 4 ratio) in solution after mixing for 25 ms, 1 s, and 5 s.

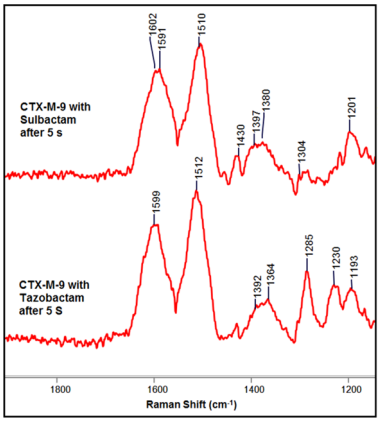

Figure 3. Raman difference spectra of CTX-M-9 with sulbactam and tazobactam (1 to 4 ratio) in solution after 5 s.

time points (100 ms and 1 s, Figures S3 and S4, Supporting Information) shows that the population of acrylate species increases from 100 ms to 5 s.

The slight variation in the exact position of "acrylic species" signature between Figures 2 and 3, reflects the fact that the inhibitor−enzyme contacts vary for all three inhibitors and must lead to small differences in torsional angles in the β-alkoxyacrylate framework. The reproducibility of these data, and of other results, is confirmed by Figures S1−S5.

Comparing these results to the previous studies on the reaction of SHV-1 and three inhibitors in crystals and aqueous solution,[9,13,21] we see that formation of cross-linked species and subsequent hydrolysis product (Scheme 1, species 4 and 6) occurs less frequently among other β-lactamase enzyme in class A such as TEM and SHV.[13,18,20−23] In the case of CTX-M-9, we observe a major population of β-alkoxyacrylate species on the time scale of minutes when the enzyme reacting with inhibitors in a single crystal and when the enzyme−inhibitor reaction occurs in aqueous solution, the cross-linked species forms very quickly (less than 25 ms). To the best of our knowledge, this is the first structural study that shows cross-linking occurs rapidly in solution in a reaction between a β-lactamase enzyme and each of the three inhibitors, which might be a major cause of inhibition *in vivo*.

Formation of the cross-linked species can also explain why a substitution at S130 produces inhibitor resistance[26] in this class of enzymes. To further test this, we used a variant of CTX-M-9 in which serine 130 was replaced by glycine (S130G). Using a rapid mix−rapid freeze technique,[21] the CTX-M-9 S130G reactions with clavulanic acid, sulbactam, or tazobactam were studied in aqueous solution, and Figure 4 shows the Raman difference

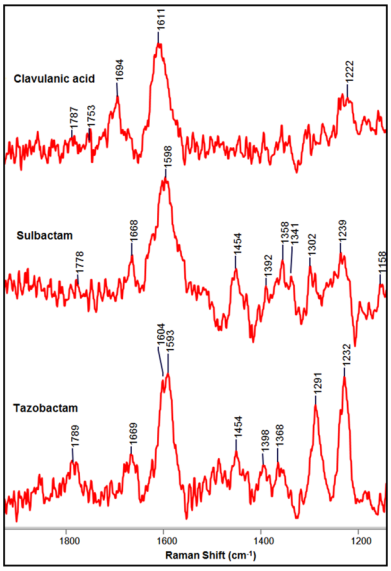

Figure 4. Raman difference spectra of CTX-M-9 S130G with clavulanic acid, sulbactam, or tazobactam (1 to 4 ratio) in solution after 5 s. The appearance of features at 1694 cm^{-1} (clavulanic acid) and 1789 cm^{-1} (tazobactam) indicates the presence of unreacted inhibitors. This is consistent with the S130G variant being less reactive than the wild type enzyme.

spectra of freeze-dried reaction intermediates 5 s after mixing. Comparison of the Raman difference spectra of CTX-M-9 wild type (Figures 1−3) and variant enzyme, S130G (Figure 4) with the three inhibitors, clearly shows an important difference. The absence of a peak around 1520 cm^{-1} and presence of 1600 cm^{-1} in Raman difference spectra shown in Figure 4 indicate that *trans*-enamine is the major intermediate formed in CTX-M-9 S130G reactions and that the β-alkoxyacrylate species cannot be detected. The intensities of Raman bands are directly proportional to the population of the molecules of the parent.[27] In the present case, by comparing the enamine peak in Figures 3 and 4, we estimate that the relative population of "acrylate species" to enamine species must be decreased by approximately a factor of 10 for the acrylate to be undetectable in Figure 4. The data in Figure 4 was repeated for different focal spots in the lyophilized samples (e.g., Figure S5, Supporting Information). Again there is no detectable feature near 1520 cm^{-1}. In the wt enzyme reaction, a large population of the cross-linked species occurs very quickly, in less than 1 s, in solution. Since the β-alkoxyacrylate species is formed essentially irreversibly, this is likely to be a major factor in the enzyme inhibition. Since this cannot occur for the S130G variant, the data suggests that this substitution will give rise to a higher IC_{50} (50% inhibitory concentration) compared to that for wt enzyme. The *in vitro* studies show 40- to 650-fold increase in IC_{50} for the CTX-M-9 S130G mutant to clinical inhibitors

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(clavulanic acid, sulbactam, and tazobactam)[26] compared to the wt enzyme.

Although the natural inhibitor-resistant variants of β-lactamases (S130G) have been described among SHV and TEM β-lactamases,[28−30] the natural inhibitor resistant of CTX-M S130G has not yet been reported. However, *in vitro* evolution studies show that the S130G mutation is one of the most common and provides a high-level resistance to conventional β-lactamase inhibitors.[31,32]

There are several hypotheses that would explain the fast formation of cross-link species in the reaction of CTX-M-9 enzyme with the inhibitors. Structural studies[4,33] show two conformations for lysine 73 in the active site of CTX-M-9. In conformation 1, Lys73 is too far to make a hydrogen bond with Ser130Oγ, whereas in conformation 2, Lys73 rotates and establishes a hydrogen bond with Ser130. We hypothesize that as a result of conformation 1, Ser130Oγ is less involved with hydrogen bonding interactions and can act more readily as a nucleophile to attack protonated imine to form a cross-link. The second hypothesis relies on the higher dynamic excursion in the active site of CTX-M family compared to SHV and TEM. Previous studies suggest that protein flexibility and enhanced mobility can lead to an extended substrate range among CTX-M enzymes.[4,6] We hypothesize that enhanced mobility in the active site of CTX-M-9 leads to a higher population where there are transient interactions between Ser130 and the imine intermediates. In turn, these increase the probability of Ser130Oγ nucleophilic attack at the imine thereby increasing the likelihood of forming a cross-linked species.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b10007.

Details of experimental procedures and Figures S1−S5 (PDF)

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Notes

The authors declare no competing financial interest.

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