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Raman Spectra of Interchanging β-Lactamase Inhibitor Intermediates on the Millisecond Time Scale

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Supporting Information

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 and then were flash-frozen by injection into an isopentane solution surrounded by liquid nitrogen. The "ice" was finally freeze-dried and characterized by Raman microscopy. We found that the reaction is almost complete in solution at 25 ms, giving rise to a major population composed of the *trans*-enamine intermediate. Between 25 and 500 ms, minor populations of protonated imine are detected that have previously been postulated to precede enamine intermediates. However, within 1 s, the imines are converted entirely to enamines. Interestingly, with this method, we can measure directly the turnover number of SHV-1 and tazobactam. The enzyme is completely inhibited at 1:4 ratio (enzyme:inhibitor) or greater, 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.

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.[1,2] Another approach is to switch on the reaction by excitation from a pulse of light and then probe the intermediate with ultrafast spectroscopic tools.[3,4] These techniques, however, are limited to specific reaction systems. With the advent of the rapid mix−rapid quench technique, the pace of studying metastable reaction intermediates accelerated significantly. The continuous improvement of mixers has made this technique an attractive tool because of its short time scales and low sample consumption.[5−9] In the freeze-quench technique, first introduced by Bray in 1961, the mixed reactant solution is rapidly frozen by low-temperature isopentane (−130 °C).[10]

Raman or resonance Raman spectra of reaction intermediates in water have the potential of providing a plethora of information on enzyme mechanisms.[11−13] In 2003, Lin et al. combined ultrafast mixing with a rapid freeze-quench device and could detect intermediates as early as 50 μs.[14] 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, we obtained high-quality Raman difference spectra of the freeze-dried material in the presence and in the 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 were able to trap the intermediate at 25 ms and longer after mixing using ∼0.5 mg of protein at each time point.

β-Lactamases confer antibiotic resistance to bacteria by hydrolyzing antibiotic drugs such as penicillins and cephalosporins.[15,16] To date, more than 700 unique β-lactamases have been reported.[17] According to their amino acid sequences (Ambler method), β-lactamase enzymes are generally divided into four classes, A−D.[18,19] Among the well-studied class A enzymes, SHV-1 and TEM-1 are the most commonly encountered 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.[20] Unfortunately, bacteria also acquire resistance to these inhibitors due to mutations in β-lactamases.

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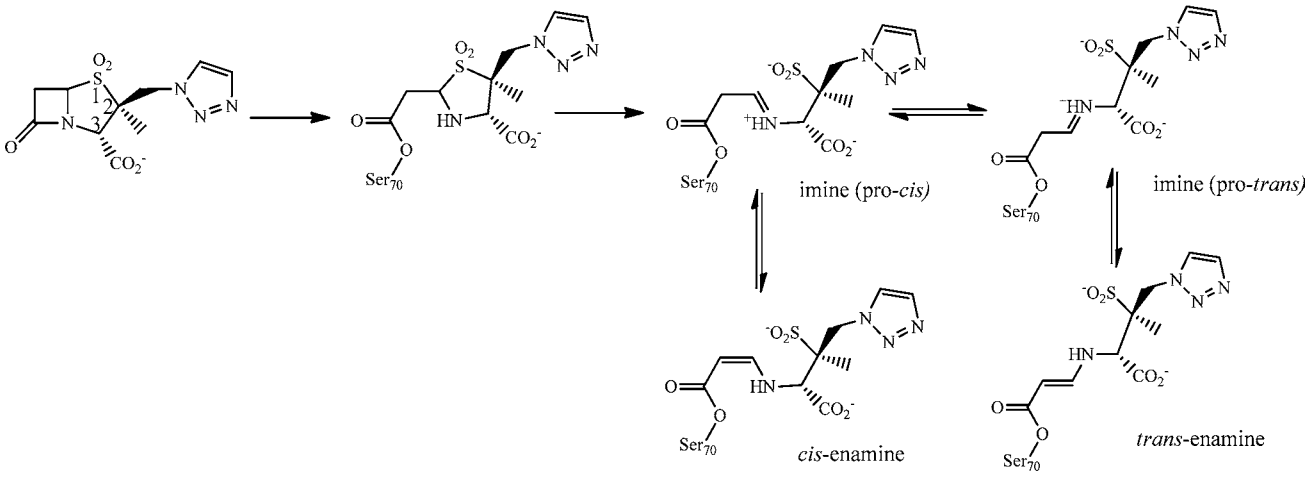
Scheme 1. Partial Reaction Scheme for Tazobactam and Class A β-Lactamase Enzymes



For a decade, our laboratory has developed Raman crystallography to track reactions of the clinical inhibitors in single crystals of β-lactamase in real time.[20] 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 usually depends 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.

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 with this reaction in single crystals,[20−26] and here we compare the reaction intermediates formed in crystal and solution environments. A partial reaction scheme is shown in Scheme 1.

When following the reaction in a single crystal of wild-type SHV-1, we showed that there is an equilibrium between the major populations of protonated imines, *cis*- and *trans*-enamine (Scheme 1).[20,26] The Raman difference spectrum for tazobactam reacting in a single crystal of SHV-1 β-lactamase is shown in Figure 1. The crucial underpinnings of the band assignments came from combined Raman and X-ray crystallographic experiments. On the basis of 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=C−NH− fragment of *trans*-enamine.[27] This prediction and the presence of the *trans*-enamine species was confirmed by subsequent X-ray analysis.[28,29] Detailed analysis from Gaussian calculations (see Table 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 1).[20]

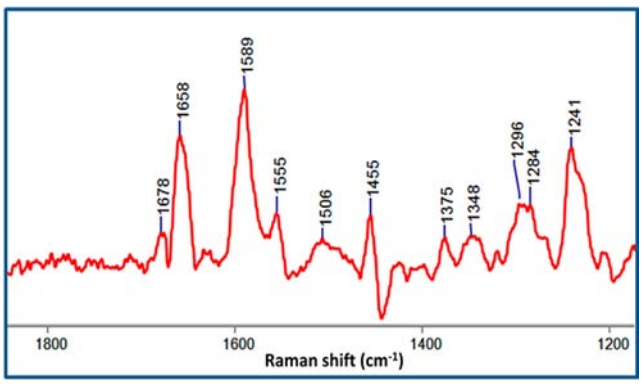


Figure 1. Partial Raman difference spectrum of the intermediate from the tazobactam reaction with SHV-1 in a single crystal after 20 min of soaking in 20 mM tazobactam.

In Figure 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 *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-*cis* imine. Kalp et al.[20] assigned it to an amide I feature; however, the present band is too narrow for us to unambiguously make this assignment. Similarly, it is possible that the profile at 1589 cm^{-1} also contains a contribution from *cis*-enamine.

The tazobactam triazolyl ring has a fairly intense mode near 1290 cm^{-1} in aqueous solution (see Figure 2 below, upper trace). In Figure 1, this mode appears to have multiple components, which we ascribe 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 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.

Before comparing the results between *in crystallo* and 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 (Supporting Information, Figure S1) are very similar. The similarities in the amide I (1660 cm^{-1} region) and 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 around 530 cm^{-1}. The latter is due to the S–S mode involving cysteine residues at positions 77 and 123,[30] and the S–S stretch mode is sensitive to changes in conformation about these linkages.[31] The small differences in Figure S1 are probably due to minor changes in loops and disordered sequences occurring upon dehydration.

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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 in the presence of 1 or 10 μM 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 S2 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.

Figure 2 compares the Raman difference spectra for flash-frozen acyl enzyme intermediates, between 25 and 1000 ms after mixing, with the spectrum of free aqueous tazobactam (upper trace). For the intermediates, the broad band near 1595 cm^{-1} (~45 cm^{-1} 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 1). Kalp et al.[20] suggested the similar sulbactam intermediates to have enamine marker bands at 1588 and 1605 cm^{-1}, respectively. The sharp triazole peak at 1287 cm^{-1} (19 cm^{-1} width at half-height) indicates that the "natural line width" (of sharp features) in the lyophilized powder is ~20 cm^{-1}, and is evidence that the broad line width of 1596 cm^{-1} in Figure 2 may be due to multiple isomers. The protonated imine peaks at 1658 (pro-*trans*) and 1673 cm^{-1} (pro-*cis*) in Figure 2 are narrow and, if assigned correctly, suggest a higher relative population of the pro-*trans* imine isomer in solution compared to pro-*cis*.

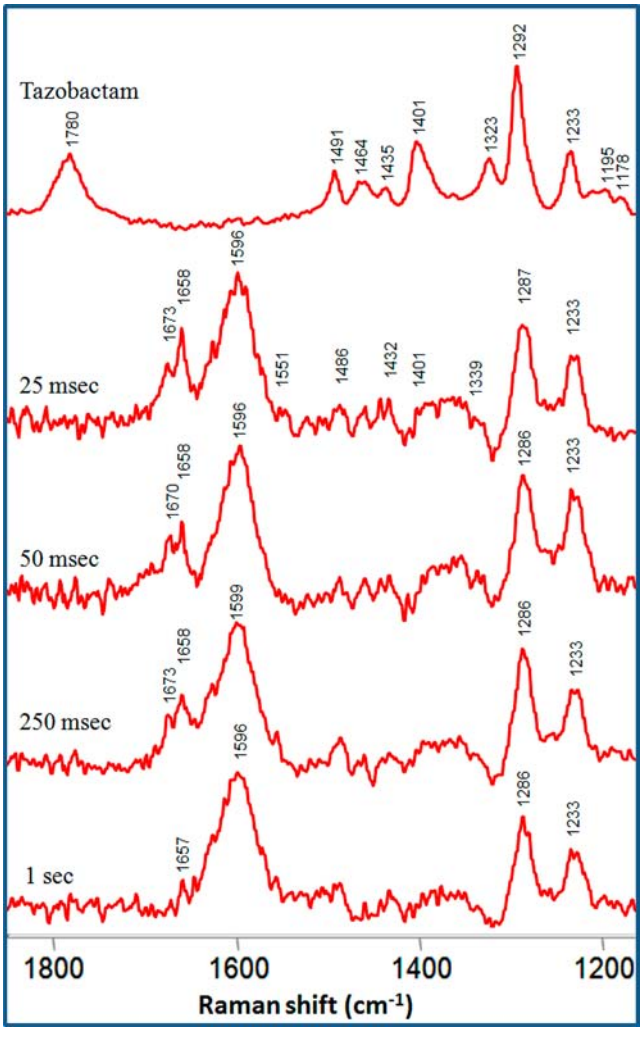

Figure 2. Partial Raman spectrum of tazobactam (20 mM, pH 7.0, top) and Raman difference spectra of tazobactam reacting with SHV-1: trapped intermediates in lyophilized powder between 25 and 1000 ms after mixing. In the tazobactam spectrum, the 1780 cm^{-1} feature corresponds to the stretch of the carbonyl group in the β-lactam ring.

Table 1. Raman Peak Assignments for the Major Peaks in Tazobactam/SHV-1 Difference Spectra Shown in Figure 2

species	wavenumber (cm^{-1})	peak assignment
unreacted tazobactam	1780	C═O of β-lactam ring
	1491	C═C of triazolyl ring
	1401	CO_2^- (symmetric stretch) at C3 position
	1292	breathing of triazolyl ring
	1233	N═N of triazolyl ring
imine	1658	C═NH$^+$ of imine
trans-enamine	1596	−O−C(═O)−C═C−NH− stretch of *trans*-enamine

The relative population of pro-*trans* imine in the crystal (Figure 1) appears to be higher compared to the population of pro-*trans* imine in solution (Figure 2), and *in crystallo*, the intense imine peak remains at 60 min. Since in solution the imine feature almost disappears at 1 s, the "lifetime" of the imine in the crystal is >10^3 times longer than in solution. Thus, in solution, at 1 s after mixing, the imine isomers seem to have essentially converted to enamine. We have detected the formation of enamine isomers quickly, within 25 ms after mixing, that remain essentially unchanged after 1 s. These isomers are accompanied by the expected imine predecessors that convert to enamine within 1 s.

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 s of mixing by injection 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 the original spectrum (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 3. The enamine peak intensity is constant after reaching a ratio of 1:4−1:5, which shows that the turnover number is between 4 and 5, which agrees with the value of 5 obtained from kinetic methods.[32]

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 the Raman signal is enhanced by freeze-drying the sample. The results due to the binding of inhibitor to the

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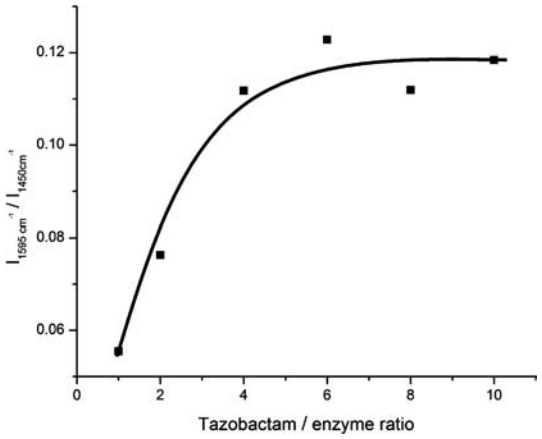

Figure 3. Direct measurement of turnover number for tazobactam reaction with SHV-1 enzyme. The enzyme is completely inhibited, giving rise a maximum enamine population after 4−5 equiv of tazobactam has been hydrolyzed.

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.[33] The method outlined herein should be widely applicable to other enzyme−substrate reaction.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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