Clavulanic Acid Inactivation of SHV-1 and the Inhibitor-resistant S130G SHV-1 β-Lactamase

INSIGHTS INTO THE MECHANISM OF INHIBITION

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Clavulanic acid is a potent mechanism-based inhibitor of TEM-1 and SHV-1 β-lactamases, enzymes that confer resistance to β-lactams in many Gram-negative pathogens. This compound has enjoyed widespread clinical use as part of β-lactam β-lactamase inhibitor therapy directed against penicillin-resistant pathogens. Unfortunately, the emergence of clavulanic acid-resistant variants of TEM-1 and SHV-1 β-lactamase significantly compromises the efficacy of this combination. A single amino acid change at Ambler position Ser70 (Ser → Gly) results in resistance to inactivation by clavulane in the SHV-1 and TEM-1 β-lactamas. Herein, we investigated the inactivation of SHV-1 and the inhibitor-resistant S130G variant β-lactamases by clavulane. Using liquid chromatography electrospray ionization mass spectrometry, we detected multiple modified proteins when SHV-1 β-lactamase is inactivated by clavulane. Matrix-assisted laser desorption ionization-time of flight mass spectrometry was used to study tryptic digests of SHV-1 and S130G β-lactamases (± inactivation with clavulane) and identified peptides modified at the active site Ser70. Ultraviolet (UV) difference spectral studies comparing SHV-1 and S130G β-lactamases inactivated by clavulane showed that the formation of reaction intermediates with absorption maxima at 227 and 280 nm are diminished and delayed when S130G β-lactamase is inactivated. We conclude that the clavulanic acid inhibition of the S130G variant β-lactamase must follow a branch of the normal inactivation pathway. These findings highlight the importance of understanding the intermediates formed in the inactivation process of inhibitor-resistant β-lactamases and suggest how strategic chemical design can lead to novel ways to inhibit β-lactamases.

Among Gram-negative bacteria, β-lactamase enzymes (EC 3.5.2.6) are the principal agents of bacterial resistance to penicillin and cephalosporin antibiotics. β-Lactamases hydrolyze β-lactams and render them ineffective before they reach their targets, the penicillin-binding proteins. This two-step process requires the presence of a strategically located water molecule in the active site (1). To combat the critical problem of β-lactamase-mediated resistance, two approaches were undertaken: design β-lactams resistant to the hydrolytic action of β-lactamases or find inhibitors of these enzymes (2–5). Currently, tazobactam, sulbactam, and clavulanic acid are the only β-lactamase inhibitors used in combination with β-lactams for the treatment of infections by bacteria that possess class A β-lactamases (Fig. 1) (6). Administered with a β-lactam, β-lactamase inhibitors (e.g. ampicillin/sulbactam, amoxicillin/clavulane, piperacillin/tazobactam, cefporenzone/sulbactam, and ticarcillin/clavulane) have had a significant impact on the treatment of a wide variety of infections.

TEM-1 and SHV-1 are class A β-lactamases commonly found in Escherichia coli and Klebsiella pneumoniae, pathogens responsible for urinary tract, respiratory tract, and bloodstream infections (6). In the past decade, single amino acid substitutions in these enzymes have given rise to β-lactamases that are resistant to inactivation by inhibitors (the inhibitor-resistant TEMs). To date, 22 TEM and 3 SHV β-lactamases are described in nature that confer this phenotype (www.lahey.org/studies/webt.asp) (7–13). Atomic structure determinations of the inhibitor-resistant TEMs (M69V, M69L, M69I, R244S, and N276D TEM β-lactamases) reveal the importance of understanding how subtle changes have a profound impact on inhibitor resistance (11–13). However, many of the molecular and structural details of β-lactamase inactivation remain secret. To address this, the inactivation of variant SHV β-lactamases has been studied using multiple approaches (14–18).

The clinically relevant and unique S130G variant of SHV-1 β-lactamase is 60-fold more resistant to inactivation by the inhibitor tazobactam, and 330-fold more resistant to clavulane than SHV-1, as defined by KI (18). Despite a significant decrease in affinity for inhibitors and a decrease in efficiency of inactivation (kmax/ki, 100-fold for tazobactam and 420-fold for clavulane), the S130G enzyme is eventually inhibited by both compounds. Our previous mass spectrometry studies of SHV-1 and SHV S130G β-lactamases with tazobactam suggested that a hydrated aldehyde (Δ + 88) was an important intermediate formed in the inactivation of SHV-1, whereas an aldehyde (Δ + 70) was the predominant intermediate in the inactivation of S130G. Because we saw higher resistance to inactivation for S130G with clavulante, we asked whether inactivation by this compound occurs via an alternate pathway to the one followed by SHV-1 β-lactamase.

In this report, we continue our analysis of the S130G substitution and examine the inactivation of S130G and SHV-1 β-lactamases with clavulane.
vulanate. Using liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS), we show that different mass adducts are formed when SHV-1 and S130G are inactivated by clavulanate. These data support the hypothesis that inhibitor-resistant class A \(\beta\)-lactamases follow different reaction pathways and kinetics on their way to inactivation when compared with their inhibitor susceptible counterparts (18). The importance of understanding changes in the active site structure as it relates to the formation of reaction intermediates in \(\beta\)-lactamase inhibitor design is assessed.

MATERIALS AND METHODS

Preparation and Purification of SHV-1 and S130G-substituted \(\beta\)-Lactamases—The \(E.\ coli\) DH10B strains possessing the SHV-1 and S130G \(\beta\)-lactamases were previously described (19). In all instances, purified SHV-1 and S130G \(\beta\)-lactamases were prepared by isoelectric focusing using an established method (15). The purification and storage buffers were 20 mM diethanolamine, pH 8.5.

Inactivation of SHV-1 and S130G-substituted \(\beta\)-Lactamases by Clavulanic Acid for LC-ESI/MS and MALDI-TOF/MS Studies—Lithium clavulanic acid, a kind gift from Glaxo Smith Kline (Research Triangle Park, NC), was dissolved in high performance liquid chromatography grade water and mixed with the study \(\beta\)-lactamases in a molar ratio of 1000:1 (inhibitor:enzyme) at room temperature for 30 min. We chose this ratio of inhibitor, \(I\), to enzyme, \(E\), to be consistent with our previous studies with tazobactam and to ensure complete inactivation (18). Our time of inactivation, 30 min, approximates the generation time of \(E.\ coli\).

Next, the inhibited enzymes were analyzed by the LC-ESI/MS method described herein.

The LC-ESI/MS analyses were performed using a Micromass (Beverly, MA) QUATTRO II triple quadrupole mass spectrometer with electrospray ionization interfaced to an Agilent (Colorado Springs, CO) 1100 series high performance liquid chromatography system as described previously (18). A C\(_4\) reverse phase column (1 \(\times\) 150 mm, 5 \(\mu\)m, 300 Å) purchased from Vydac (Hesperia, CA) was used to purify each apoenzyme and inactivated \(\beta\)-lactamase from unbound clavulanate (molecular mass = 199.3 Da). The mobile phase was a gradient mixture of A (0.3% acetic acid in water) and B (0.3% acetic acid in acetonitrile) with a flow rate through the column of 45 \(\mu\)l/min. The gradient timetable was 2% B at 0 min and 80% B at 30 min, and the total run time was 60 min. The parameters for the LC-ESI/MS were: ionization mode, positive; cone voltage, 70 V; \(N_2\) used both as a nebulizing and as a drying gas; the source temperature, 70 °C; capillary voltage, 3.5 KV, and the spectra were obtained scanning over 800–2400 Da. The Omnimflex MALDI-TOF/MS from Bruker (Billerica, MA) was also used (see below).

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FIGURE 1. Class A \(\beta\)-lactamase inhibitors in clinical use.

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FIGURE 2. a, LC-ESI/MS of SHV-1 \(\beta\)-lactamase. b, LC-ESI/MS of SHV-1 \(\beta\)-lactamase inactivated with clavulanate. Multiple peaks are identified.

FIGURE 3. a, LC-ESI/MS of S130G \(\beta\)-lactamase. b, LC-ESI/MS of S130G \(\beta\)-lactamase inactivated by clavulanate.

Proteolysis—The uninhibited and clavulanic acid-inactivated \(\beta\)-lactamases were first denatured with 8 M urea at 50 °C for 30 min. The pH of the denaturation mixture was adjusted to 8 using 0.4 M \(NH_4HCO_3\). After a 1:4 dilution with water, sequencing grade trypsin (Promega) was

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4 The abbreviations used are: LC-ESI MS, liquid chromatography-electrospray ionization mass spectrometry; MALDI-TOF/MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.
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**TABLE ONE**

| Peptides identified by LC-ESI/MS | Mass by theoretical trypsin digest | Amino acid residue assignments | Peptide identified by LC-ESI/MS | Mass by theoretical trypsin digest [M + H] | Peptide identified by MALDI-TOF/MS |
|---------------------------------|----------------------------------|-------------------------------|---------------------------------|------------------------------------------|----------------------------------|
|                                 |                                  |                               | Uninhibited SHV-1                |                                          | Clavulanate inhibited SHV-1      |
| 1                               | 489.2                            | 62–65                         | +                               | 490.20                                   | –                                |
| 2                               | 715.4                            | 94–98                         | +                               | 716.40                                   | +                                |
| 3                               | 716.4                            | 193–198                       | +                               | 717.42                                   | +                                |
| 4                               | 724.6                            | 216–222                       | +                               | 725.47                                   | +                                |
| 5                               | 746.4                            | 56–61                         | +                               | 747.41                                   | +                                |
| 6                               | 844.5                            | 192–198                       | +                               | 845.50                                   | –                                |
| 7                               | 874.6                            | 258–264                       | +                               | 875.57                                   | +                                |
| 8                               | 901.5                            | 154–161                       | +                               | 902.50                                   | –                                |
| 9                               | 975.5                            | 35–43                         | +                               | 976.50                                   | –                                |
| 10                              | 976.6                            | 265–273                       | +                               | 977.46                                   | +                                |
| 11                              | 987.5                            | 66–73                         | + Decreased +                   | 988.46                                   | Decreased +                     |
| 11a                             | –                                | 66–73                         | –                               | 1060.30                                   | +                                |
| 12                              | 1038.6                           | 26–34                         | +                               | 1039.58                                   | +                                |
| 13                              | 1094.6                           | 244–254                       | +                               | 1095.65                                   | +                                |
| 14                              | 1130.5                           | 84–93                         | +                               | 1131.53                                   | +                                |
| 15                              | 1277.6                           | 44–55                         | +                               | 1278.61                                   | +                                |
| 16                              | 1285.7                           | 153–164                       | +                               | 1286.70                                   | +                                |
| 17                              | 1302.6                           | 206–215                       | +                               | 1303.60                                   | –                                |
| 18                              | 1302.7                           | 223–234                       | +                               | 1303.70                                   | +                                |
| 19                              | 1334.6                           | 179–191                       | +                               | 1335.60                                   | +                                |
| 20                              | 1458.7                           | 62–73                         | + Decreased +                   | 1459.70                                   | Decreased +                     |
| 21                              | 1506.7                           | 99–111                        | +                               | 1507.72                                   | +                                |
| 21a                             |                                  |                                |                                | 1507.60                                   | –                                |
| 21b                             |                                  |                                |                                | 1520.80                                   | –                                |
| 21c                             |                                  |                                |                                | 1529.10                                   | –                                |
| 22                              | 1599.8                           | 165–178                       | +                               | 1600.7                                    | –                                |
| 23                              | 1904.8                           | 276–292                       | +                               | 1095.00                                   | +                                |
| 24                              | 1984.0                           | 162–178                       | +                               | 1985.00                                   | +                                |

**Kinetics**—The time course of inactivation and recovery of SHV-1 and S130G $\beta$-lactamases were determined by measuring residual activity at room temperature, 25 °C, using an Agilent™ 8453 Diode Array spectrophotometer. Kinetic studies were modeled after established methods (2, 3, 20). Each assay was performed using purified $\beta$-lactamase in 20 mM phosphate-buffered saline, pH 7.4, in a 1-ml quartz cuvette (2–5). Nitrocefin ($\Delta_{\text{abs}} = 17,400 \text{ M}^{-1} \text{ cm}^{-1}$) (BD Biosciences) was used as the indicator substrate. UV difference spectra (absorption spectra of clavulanate reacted with SHV-1 or S130G $\beta$-lactamase versus spectra of SHV-1 or S130G, in a 200:1 $I_{\text{E}}$ ratio) were measured from $\lambda = 190$ to 350 nm.

**RESULTS**

LC-ESI/MS and the Inhibition of SHV-1 $\beta$-Lactamase with Clavulanic Acid—SHV-1 and clavulanic acid-inhibited SHV-1 $\beta$-lactamases were first characterized using LC-ESI/MS. The deconvoluted mass spectra for the chromatographic peaks of SHV-1 and SHV-1 $\beta$-lactamase inactivated with clavulanate are shown in Fig. 2, a and b. Multiple peaks are identified in Fig. 2b. The molecular masses of the apoenzyme and clavulanate-inhibited SHV-1 $\beta$-lactamases measured by

added to the mixture at a $\beta$-lactamase:trypsin ratio of 25:1 (w/w) and incubated at 37 °C for 12 h. The mixture was frozen to −40 °C to terminate proteolysis. The digestion products were analyzed by LC-ESI/MS and MALDI-TOF/MS (18).

**LC-ESI/MS for the Tryptic Digests**—The tryptic digests of the uninhibited and clavulanic acid-inactivated $\beta$-lactamases were separated on a C18 column (1 × 150 mm, 5 $\mu$m, 300 Å) (Vydac). The mobile phase was gradient mixture of A (0.3% acetic acid in water) and B (0.3% acetic acid in acetonitrile) from 2% B to 80% B in 70 min with a flow rate through the column of 35 $\mu$l/min. The total run time was 120 min. The ESI/MS parameters were the same as described above, except that the cone voltage was 45 V and the scanning range was 300–1700 Da.

**MALDI-TOF/MS of the Tryptic Digests**—The tryptic digests were analyzed using an Omniflex MALDI-TOF/MS. Positive ionization and reflectron modes were used. The experimental parameters were as follows: laser power 60%, voltage 76.4%, and ion focus 9.2. The matrix was $\alpha$-cyano-4-hydroxycinnamic acid. The internal standard consisted of bradykinin, rennin substrate, and adrenocorticoid hormone fragments 18–39 and 7–38 (Sigma). The samples were de-salted using C18 ZipTips from Millipore before mixing with the matrix.
FIGURE 4. Expanded MALDI-TOF/MS spectra of trypsin-digested SHV-1 β-lactamase showing a decrease in fragments containing residues 66–73 and 62–73. The vertical arrow indicates reduction of a peptide peak; the slanted arrow indicates an increase in signal.

TABLE TWO

S130G β-lactamase and clavulanate inhibited S130G β-lactamase peptides identified with LC-ESI/MS and MALDI-TOF/MS

For S130G, 24 peptides were identified and mass determined by LC-ESI/MS. When S130G β-lactamase is inactivated by clavulanate, 22 peptides are identified by LC-ESI/MS. Eighteen peptides were assigned by MALDI-TOF/MS. When S130G is inactivated by clavulanate, 16 peptides were identified. The amino acid assignments were made as in Table I. Protein Prospector displayed peptides with mass greater than 500 Da. “+” means peptide found; “−” means peptide absent.

| Peptides identified by LC-ESI/MS | Mass by theoretical trypsin digest | Amino acid residue assignments | Peptide identified by LC-ESI/MS | Mass by theoretical trypsin digest [M + H] | Peptide identified by MALDI-TOF/MS |
|----------------------------------|-----------------------------------|-------------------------------|---------------------------------|-------------------------------------------|---------------------------------|
|                                  |                                   |                               | Uninhibited S130G | Clavulanate-inhibited S130G | Uninhibited S130G | Clavulanate-inhibited S130G |
| 1                                | 489.2                             | 62–65                         | +                  | +                          | 490.20                          | −                               |
| 2                                | 715.4                             | 94–98                         | +                  | +                          | 714.60                          | +                               |
| 3                                | 716.4                             | 193–198                       | +                  | +                          | 717.42                          | +                               |
| 4                                | 724.5                             | 216–222                       | +                  | +                          | 725.47                          | +                               |
| 5                                | 746.4                             | 56–61                         | +                  | +                          | 747.41                          | +                               |
| 6                                | 844.5                             | 192–198                       | +                  | +                          | 845.50                          | −                               |
| 7                                | 874.6                             | 258–264                       | +                  | +                          | 875.57                          | +                               |
| 8                                | 901.5                             | 154–161                       | +                  | +                          | 902.46                          | −                               |
| 9                                | 975.5                             | 35–43                         | +                  | +                          | 976.50                          | −                               |
| 10                               | 976.4                             | 265–273                       | +                  | +                          | 977.44                          | +                               |
| 11                               | 987.5                             | 66–73                         | +                  | −                          | 988.46                          | −                               |
| 12                               | 1038.6                            | 26–34                         | +                  | +                          | 1039.58                         | +                               |
| 13                               | 1094.6                            | 244–254                       | +                  | +                          | 1095.65                         | +                               |
| 14                               | 1130.5                            | 84–93                         | +                  | +                          | 1131.53                         | +                               |
| 15                               | 1277.6                            | 44–55                         | +                  | +                          | 1278.61                         | +                               |
| 16                               | 1285.7                            | 153–164                       | +                  | +                          | 1286.70                         | +                               |
| 17                               | 1302.6                            | 206–215                       | +                  | +                          | 1303.64                         | −                               |
| 18                               | 1302.7                            | 223–234                       | +                  | +                          | 1303.70                         | −                               |
| 19                               | 1334.7                            | 179–191                       | +                  | +                          | 1335.65                         | +                               |
| 20                               | 1458.7                            | 62–73                         | +                  | −                          | 1459.70                         | −                               |
| 21                               | 1506.7                            | 99–111                        | +                  | +                          | 1507.72                         | +                               |
| 22                               | 1599.8                            | 165–178                       | +                  | +                          | 1600.76                         | −                               |
| 23                               | 1904.0                            | 276–292                       | +                  | +                          | 1905.00                         | +                               |
| 24                               | 1984.0                            | 162–178                       | +                  | +                          | 1985.00                         | +                               |
LC-ESI/MS are: 28,874 ± 3 Da (SHV-1); 28,923 ± 3 (+Δ 49 ± 6); 28,946 ± 3 Da (+Δ 72 ± 6); 28,962 ± 3 (+Δ 88 ± 6); 28,987 ± 3 (+Δ 113 ± 6); and 29,048 (+Δ 174 ± 6). These additional masses are less than the molecular mass of clavulanic acid (199.3 Da) directly added to SHV-1, indicating that fragments of the inhibitor are covalently attached and modified in the clavulanic-SHV-1 complexes. The mass increments are very reminiscent of the increases seen in the clavulanic acid inactivation of TEM-2 by Brown et al. (21) (+Δ 52, 70, 88, and 155 Da), the tazobactam inactivation of TEM-1 and PC1 β-lactamases by Yang et al. (22) (+Δ 52, 70, 88 Da), and the N276D variant of TEM-1 by clavulanate (+Δ 88 Da) (10). We note that two of the products (113 ± 6 and 174 ± 6 Da adducts) were not identified in the above studies or with the inactivation of SHV-1 or S130G β-lactamases by tazobactam (18).

LC-ESI/MS and the Inhibition of S130G β-lactamase by Clavulanic Acid—S130G and clavulanic acid-inhibited S130G variants of SHV-1 β-lactamase were studied in the same manner as the SHV-1 apoenzyme. Using LC-ESI/MS, we determined that the S130G β-lactamase had a uniform mass increase of 70 ± 6 Da from 28,839 to 28,909 Da (Fig. 3, a and b) after inhibition with clavulanic acid. This is the identical mass increase seen with the tazobactam inactivation of S130G β-lactamase (18).

Identifying the Amino Acid Residues Modified by Clavulanic Acid in SHV and S130G β-Lactamase Using Tryptic Digestion and MALDI-TOF/MS Analysis—To gain insight into the mechanism and pathway of inactivation, the apo-form and clavulanic acid-inhibited SHV-1 and S130G β-lactamases were digested with trypsin, and the tryptic digests were analyzed with LC-ESI/MS and MALDI-TOF/MS. Our goal here was to identify the amino acid site(s) of modification in the SHV-1 and S130G β-lactamases.

Twenty-four peptides were identified by LC-ESI/MS from the tryptic digest of SHV-1 and 25 peptides were identified by LC-ESI/MS of SHV inhibited by clavulanic acid (TABLE ONE). Using the theoretical digestion of SHV-1 β-lactamase as a guide, we assigned 18 peptides by MALDI-TOF/MS of SHV-1 digested by trypsin, whereas 22 peptides were assigned by MALDI-TOF/MS analysis of SHV-1 inactivated by clavulanate (TABLE ONE). Fig. 4, a and b, shows the expanded MALDI-TOF/MS spectra for the tryptic digest of the apo-SHV-1 and clavulanate-inhibited SHV-1 β-lactamase with signals from the [M + H] ions of 988.46 and 1459.70, respectively. As we observed in the MALDI-TOF/MS analysis of SHV-1 inactivated by tazobactam, the relative signal intensities of peptide 11 that contains residues 66–73 (M + H, 988.46) and peptide 20 spanning residues 62–73 (M + H, 1459.70) were significantly decreased after clavulanic acid inhibition of SHV-1 β-lactamase. We noted the appearance of a peak (M + H, 1060.30) in the MALDI-TOF/MS for the digest of the clavulanate-inhibited SHV-1 that was not evident in the apo-SHV-1 β-lactamase analysis (Fig. 4a, TABLE ONE, peptide 11a). The mass difference between this peak and the diminished peptide peak at 988.46 m/z (residues 66–72) is 72 Da and is in agreement with one of the mass increases of SHV-1 after inhibition by tazobactam (see also LC-ESI/MS spectra above). For the peptide spanning amino acids 62–73 (M + H, 1459.70, peptide 20), the decrease in intensity is accompanied by the appearance of three new mass increments (M + H, 1507.60, 1520.80, and 1529.10) (pep-
tides 21a, 21b, and 21c, respectively) (Fig. 4b, TABLE ONE). These mass increases correspond to: 47.90 Da, peptide 21a; 61.10 Da, peptide 21b; and 69.40 Da, peptide 21c. Two of the mass increments (Δ47.90 and 69.40) are in close agreement with the LC-ESI/MS determination (49 ± 6 and 72 ± 6 Da); the nature of the 61.10-Da intermediate remains unexplained.

FIGURE 6. a, UV difference spectra of clavulanate inactivating SHV-1 β-lactamase in a 200:1 I:E ratio for 1 h. The peak absorption at λ = 280 nm occurs at 15 min and is diminished by 60 min. The peak absorption at λ = 227 nm occurs at 12 s and diminishes by 60 min. b, UV difference spectra of clavulanate inactivating S130G β-lactamase in a 200:1 I:E ratio for 1 h. The peak absorption at λ = 280 nm occurs at 60 min and is delayed and decreased in intensity compared with SHV-1 inactivated with clavulanate under similar conditions. The peak absorption at λ = 227 nm was absent.

FIGURE 7. Time course of inactivation and recovery of enzymatic activity. Thirty nm SHV-1 and S130G β-lactamase are inactivated by clavulanate for 24 h (20:1 I:E ratio).
A different pattern is seen with the LC-ESI/MS and MALDI-TOF/MS studies of S130G inactivated with clavulanate. In the digests containing S130G and S130G inhibited by clavulanic acid, 24 peptides were also identified by LC-ESI/MS for S130G and 22 peptides were seen in the digest of S130G inactivated by clavulanate (TABLE TWO). By MALDI-TOF/MS, 18 peptides were assigned after digestion of the apo-S130G and 16 were assigned in the clavulanate-inhibited S130G β-lactamase. The expanded MALDI-TOF/MS of trypsin-digested S130G showed a decrease in digested fragments containing residues 66–73 (M+H, 988.40) and 62–73 (M+H, 1459.70) (Fig. 5, a and b). Taken together, these data are consistent with the observation that the modification sites of SHV-1 and S130G β-lactamase inactivated by clavulanate are primarily located between residues 66–73 and 62–73 of the SHV-1 and S130G β-lactamase (at the active site Ser70). A limitation here is the inability of MALDI-TOF/MS to demonstrate a corresponding increase in peptide signal intensity as with SHV-1 inactivated with clavulanate (Fig. 4, a and b).

**UV Difference Spectra and Measurement of Residual Activity**—UV difference spectra of the inactivation of SHV-1 and S130G β-lactamase for 60 min (200:1, I:E ratio) demonstrated the appearance of a peak at 280 nm consistent with the formation of the β-aminoacrylate or enamine intermediate (2, 20). This peak was noticeably
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**DISCUSSION**

**Insights into the mechanism of inactivation of SHV-1 and S130G β-Lactamases by Clavulanate**—Under these conditions, the inactivation of the inhibitor-susceptible SHV-1 enzyme by clavulanate yielded different intermediates than we observed with tazobactam (18). Our data examining SHV-1 are reminiscent of other analyses (10, 21, 22) and clearly show that many intermediates are formed during the 30 min of inactivation. In distinction, only a single adduct is found when S130G β-lactamase is inactivated by clavulanate under these conditions. This was evident using LC-ESI/MS and MALDI-TOF/MS. Interpretation of these data in light of established paradigms allows us to propose the following contrasting mechanisms to explain the pathway to inactivation of SHV-1 and S130G β-lactamase by clavulanate (21–23).

In the case of SHV-1 β-lactamase inactivated by clavulanate, the Ser⁷⁰-OH of SHV-1 attacks the carbon of the carbonyl group in the β-lactam ring (Fig. 8). This leads to the acyl-enzyme 4 that undergoes further reaction to 5 to generate a linear imine species 6 (highlighted in red) after oxonium ring opening and departure of the oxygen from C-5. This imine proceeds next to a cis- and trans-enamine (β-aminoacyclate), 7 and 11, via rearrangement. Based upon earlier studies, we propose that the absorption peak at 280 nm represents isomerization to the trans-enamine (cis-enamine peak at λ = 295 nm, trans-enamine at λ = 280) (24, 25). Formation of the trans-enamine is favored because it relieves the strain imposed upon the first and fourth atoms defining the cis-double bond, the carbonyl oxygen atom (25).

From the intermediate 6, an aldehyde can also be formed 13 (Δ+70). This aldehyde can be hydrated to 14 (Δ+88). Alternatively, 13 may react with Ser¹⁵⁰-OH to yield the putative “bridged species,” 15 (Δ+52) by proceeding through a hemiacetal 13a (21). Intermediate 16 is next formed with hydration of the bridged species. Intermediate 16 would be reminiscent of the intermediate seen in the inactivation of SHV-1 by tazobactam.

As shown, the decarboxylated imine 12a (Δ+156) can tautomerize to 12b, and be hydrated to two different intermediates, 12c and 12d (Δ+174). Hydration of 12d yields 13 (the aldehyde) directly.

In an alternative path, the cis-enamine 7 may decarboxylate to form 8. This could be assisted by an active site Lys. The decarboxylated cis-enamine can exist in equilibrium with the decarboxylated imine 9. Alternatively, the decarboxylated imine can be generated from the enolate 6a. If an active site Lys would accept the −CO₂ (Δ+44) and an aldehyde is formed (Δ+70), we would account for the Δ+113 ± 6 adduct. The intermediate 10 (aldehyde at Ser⁷⁰ with a carbamylated Lys) could be formed by this route.

These pathways account for the intermediates seen in our LC-ESI/MS studies (highlighted in blue, Fig. 8). In interpreting the observed molecular masses, the acylation of the β-lactam ring results in a mass increase of 199 Da and decarboxylation reduces the adduct mass by 44 Da. No other facile fragmentation exists, except for hydrolysis of the imine forms (9 or 12a). This hydrolysis reduces the adduct mass to that of the aldehyde (Δ+70) or its hydrate (Δ+88). The observed Δ of 113 ± 6 cannot be explained by a known chemical fragmentation, but could result from the presence of a carbamylated Lys in combination with the aldehyde (10). We are cautious about this suggestion because active site Lys may be mostly protonated at pH 7.4. However, if the pKₐ of this Lys is attenuated, it is possible that the unprotonated form of Lys in solution (−NH₂) can accept a CO₂ group. Experiments are underway to explore this possibility.

Our understanding of the S130G atomic structure indicates that Ser at amino acid position 130 is not essential for enzyme inactivation (15, 26). This unorthodox consideration is fundamental to understanding the interaction of S130G with clavulanate. We propose that the pathway followed by S130G mimics SHV-1. After the acylenzyme is formed, 4, and proceeds to the imine, 6, fragmentation results and the aldehyde is formed, 13. This fragmentation is the consequence of the recruited water molecule that serves to rehydrate the active site. In addition to the proposed mechanism of inactivation of S130G with tazobactam, the water in the active site may directly attack the acyclic imine form of clavulanate (18).

The reduction of the 280 nm peak in the UV difference spectra suggests that the trans-enamine intermediate is either delayed or diminished in the S130G pathway. It has also not escaped our attention that the chromophore at 280 nm that occurs at 15 min is also within the generation time of E. coli and may represent a critical intermediate found in vivo. The structural changes resulting from the S130G substitution drive the pathway of inactivation of the inhibitor-resistant variant and either change or limit the intermediates formed. It is now clear that the path the inhibitor takes and affinity for the active site are important.

The atomic structural details of the inactivation of class A β-lactamases with clavulanate are limited to the study of *Staphylococcus aureus* penicillinase, PC1 β-lactamase (25). Cryocrystallographic studies of PC1 β-lactamase, inactivated with clavulanate revealed two species, a cis-enamine and a decarboxylated trans-enamine as trapped intermediates in the active site (25). Our investigations highlight the importance of these two intermediates. Furthermore, the carbonyl oxygen at the ester group of the former species is poorly positioned in the oxyanion hole, a finding of significant importance. Intermediates further along the reaction pathway were not seen by Chen and Herzberg (25). One wonders if each class A β-lactamase possesses singular features that dictate the pathway to inactivation. Our finding of intermediates (Δ+113 and Δ+174 ± 6 by LC-ESI/MS and 61.1 Da by MALDI-TOF/MS) not seen when TEM-2 is inactivated by clavulanate suggests this may be true.

The future development of novel β-lactamase inhibitors must build on the understanding of how amino acid changes alter catalysis. Our experiments support the notion that the clavulanic acid-resistant S130G β-lactamase follows a different branch of the normal inactivation pathway compared with wild type enzyme. In the case of the S130G β-lactamase, we assert that once the imine form of clavulanate is generated and is progressively hydrolyzed to the aldehyde, there is a lack of enzyme recovery. In contrast to this, the inhibitor-resistant SHV-1 β-lactamase “enjoys the benefit” of multiple (reversible) intermediate species and regenerates some free enzyme. This apparent “paradox of inhibitor resistance” in class A β-lactamases has significant implications (11). Recovery of free β-lactamase in a bacterium or bacterial popula-
tion possessing large amounts of SHV could affect susceptibility to \( \beta \)-lactamase inhibitors that have a rapid turnover (e.g. sulbactam), especially when cells are in a stationary phase. It is becoming clear that desirable qualities for an effective inhibitor include: permeability through the cell membrane, affinity for the active site of the target \( \beta \)-lactamase, formation of intermediates that trap the enzyme either in a “waiting room” or in a poorly hydrolysable form, displacement or elimination of a key water molecule, and prolonged time to enzyme recovery (27). Future synthetic strategies must keep in mind the multiplicity of factors that exist in this challenge and the dynamic nature of bacterial growth.

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REFERENCES

1. Helfand, M. S., and Bonomo, R. A. (2003) Curr. Drug Targets Infect. Dis. 3, 9–23
2. Charnas, R. L., Fisher, J., and Knowles, J. R. (1978) Biochemistry 17, 2185–2189
3. Charnas, R. L., and Knowles, J. R. (1981) Biochemistry 20, 3214–3219
4. Fisher, J., Charnas, R. L., and Knowles, J. R. (1978) Biochemistry 17, 2180–2184
5. Fisher, J., Charnas, R. L., Bradley, S. M., and Knowles, J. R. (1981) Biochemistry 20, 2726–2731
6. Gorbach, S. L. (1994) Intensive Care Med. 20, Suppl. 3, S27–S34
7. Knox, J. T. (1995) Antimicrob. Agents Chemother. 39, 2593–2601
8. Vakulenko, S. B., Geryk, B., Kotra, L. P., Mobashery, S., and Lerner, S. A. (1998) Antimicrob. Agents Chemother. 42, 1542–1548
9. Delaire, M., Labia, R., Samama, J. P., and Masson, J. M. (1992) J. Biol. Chem. 267, 20600–20606
10. Saves, I., Burlet-Schiltz, O., Swaren, P., Lefevre, F., Masson, J. M., Prone, J. C., and Samama, J. P. (1995) J. Biol. Chem. 270, 18240–18245
11. Swaren, P., Golemi, D., Cabantous, S., Bullychev, A., Maveyraud, L., Mobashery, S., and Samama, J. P. (1999) Biochemistry 38, 9570–9576
12. Wang, X., Minasov, G., and Shoichet, B. K. (2002) J. Biol. Chem. 277, 32149–32156
13. Meroueh, S. O., Roblin, P., Golemi, D., Maveyraud, L., Vakulenko, S. B., Zhang, Y., Samama, J. P., and Mobashery, S. (2002) J. Am. Chem. Soc. 124, 9422–9430
14. Helfand, M. S., Totir, M. A., Carey, M. P., Hujer, A. M., Bonomo, R. A., and Carey, P. R. (2003) Biochemistry 42, 13386–13392
15. Helfand, M. S., Bethel, C. R., Hujer, A. M., Hujer, K. M., Anderson, V. E., and Bonomo, R. A. (2003) J. Biol. Chem. 278, 52724–52729
16. Kuzin, A. P., Nukaga, M., Nukaga, Y., Hujer, A., Bonomo, R. A., and Knox, J. R. (2001) Biochemistry 40, 1861–1866
17. Padayatti, P. S., Helfand, M. S., Totir, M. A., Carey, M. P., Hujer, A. M., Carey, P. R., Bonomo, R. A., and van den Akker, F. (2004) Biochemistry 43, 843–848
18. Pagan-Rodriguez, D., Zhou, X., Simmons, R., Bethel, C. R., Hujer, A. M., Helfand, M. S., Jin, Z., Guo, B., Anderson, V. E., Ng, L. M., and Bonomo, R. A. (2004) J. Biol. Chem. 279, 19494–19501
19. Hujer, A. M., Hujer, K. M., and Bonomo, R. A. (2001) Biochim. Biophys. Acta 1547, 37–50
20. Bush, K., Macalintal, C., Rasmussen, B. A., Lee, V. J., and Yang, Y. (1993) Antimicrob. Agents Chemother. 37, 851–858
21. Brown, R. P., Aplin, R. T., and Schofield, C. J. (1996) Biochemistry 35, 12421–12432
22. Yang, Y., Janota, K., Tabei, K., Huang, N., Siegel, M. M., Lin, Y. I., Rasmussen, B. A., and Shlaes, D. M. (2000) J. Biol. Chem. 275, 26674–26682
23. Massova, I., and Mobashery, S. (1999) Curr. Pharm. Des. 5, 929–937
24. Cartwright, S. J., and Coulson, A. F. (1979) Nature 278, 360–361
25. Chen, C. C., and Herzberg, O. (1992) J. Mol. Biol. 224, 1103–1113
26. Savin, T., Bethel, C. R., Bonomo, R. A., and Knox, J. R. (2004) Biochemistry 43, 14111–14117
27. Nukaga, M., Abe, T., Verkatesan, A. M., Mansour, T. S., Bonomo, R. A., and Knox, J. R. (2003) Biochemistry 42, 13152–13159
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