Understanding Resistance to β-Lactams and β-Lactamase Inhibitors in the SHV β-Lactamase

LESSONS FROM THE MUTAGENESIS OF SER-130*[S]

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Bacterial resistance to β-lactam/β-lactamase inhibitor combinations by single amino acid mutations in class A β-lactamases threatens our most potent clinical antibiotics. In TEM-1 and SHV-1, the common class A β-lactamases, alterations at Ser-130 confer resistance to inactivation by the β-lactamase inhibitors, clavulanic acid, and tazobactam. By using site-saturation mutagenesis, we sought to determine the amino acid substituions at Ser-130 in SHV-1 β-lactamase that result in resistance to these inhibitors. Antibiotic susceptibility testing revealed that ampicillin and ampicillin/clavulanic acid resistance was observed only for the S130G β-lactamase expressed in Escherichia coli. Kinetic analysis of the S130G β-lactamase demonstrated a significant elevation in apparent K_m and a reduction in k_{cat}/K_m for ampicillin. Marked increases in the dissociation constant for the inhibitor to enzyme ratios revealed that ampicillin and ampicillin/clavulanic acid resistance is preserved despite amino acid substitutions that significantly alter the apparent affinity of the active site for β-lactams and β-lactamase inhibitors. These results underscore the mechanistic versatility of class A β-lactamases and have implications for the design of novel β-lactamase inhibitors.

β-Lactamase inhibition is an important strategy to combat the escalating problem of β-lactam resistance mediated by bacterial β-lactamases (EC 3.5.2.6). Combined with a β-lactam antibiotic, β-lactamase inhibitors preserve and expand the utility of the partner antibiotic and offer a greater spectrum of microbiological activity. At present, three β-lactamase inhibitors are available for clinical use: clavulanic acid, sulbactam, and tazobactam. Combined with a β-lactam antibiotic (e.g. amoxicillin/clavulanic acid, ampicillin/sublactam, ticarcillin/clavulanic acid, and piperacillin/tazobactam), these inhibitors serve as essential therapeutic agents in the treatment of life-threatening infections (1–3) (Fig. 1).

Since 1992, 23 inhibitor-resistant TEM β-lactamases and two inhibitor-resistant SHV β-lactamases have been detected (4, 5).† Investigations examining clinical isolates, using PCR and site-directed mutagenesis of blaTEM-1 and blaSHV-1, demonstrated that single amino acid substitutions at positions 69 (Met → Ile, Met → Leu, Met → Val), 130 (Ser → Gly), 244 (Arg → Ser, Arg → Cys, Arg → His, and Arg → Gly), 275 (Arg → Leu), and 276 (Asn → Asp) are the primary alterations that confer resistance to amoxicillin/clavulanic acid (7–9). High resolution atomic structures of the inhibitor resistant β-lactamases have also advanced our understanding of this phenotype. The x-ray structure of the clavulanic acid resistant N276D variant of TEM-1 β-lactamase revealed a significant movement of Asp276: the substitution of Asp for Asn displaces three helices (helix 1, helix 10, and helix 11) in the TEM structure (10). The three-dimensional analysis of the M69L TEM β-lactamase shows that subtle molecular dynamic changes can also lead to resistance to clavulanic acid (11). Most recently, the movement of Ser-130 in the inhibitor resistant TEM-30 (R244S), TEM-32 (M69I/M182T), and TEM-34 (M69V) β-lactamases has been elucidated (12).

It is particularly noteworthy that, among the inhibitor-resistant class A β-lactamases, the S130G substitution in TEM and SHV involves the conserved motif Ser-130/Asp-131/Asn-132, the “SDN” loop. Ser-130 and Asn-132 are hydrogen-bonded to the catalytically important Lys-73 in the active site. This observation challenged our understanding of the role of Gly at the 130 position in inhibitor-resistant variants of the SHV and TEM β-lactamases (e.g. SHV-10 and TEM-59) (5, 13). Exploring the consequences of amino acid substitutions on catalytic activity, we show that the apparent affinity of the active site is greatly reduced for substrates and inhibitors. In contrast, S130G and SHV-1 β-lactamases are inactivated at nearly the same rate. Our hypothesis, based on a molecular model, is that movement of a catalytic water molecule may potentially serve the same role as the hydroxyl group of Ser-130 in substrate binding and catalysis. These results have implications for the future design of β-lactams and β-lactamase inhibitors.

Received for publication, June 9, 2003, and in revised form, October 8, 2003
Published, JBC Papers in Press, October 8, 2003, DOI 10.1074/jbc.M306059200

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† Amino Acid Sequences for TEM, SHV and OXA Extended-Spectrum and Inhibitor Resistant β-Lactamases, available at www.lahey.org/studies/webt.htm.
**Experimental Procedures**

**Bacterial Strains and Plasmids**—The chromosomal SHV-1 β-lactamase with its native promoter and ribosomal binding site was cloned into the recombinant phagemid vector, pBC SK(+) (Stratagene, La Jolla, CA) as described previously (14). Plasmid DNA was obtained from *Epicurean coli* (Brатagene, La Jolla, CA) and *Escherichia coli* DH10B (Invitrogen) cells using Wizard™ Purification kits (Promega, Madison, WI).

**Mutagenesis**—PCR-based site-saturation and site-directed mutagenesis was performed using a QuickChange™ mutagenesis kit (Stratagene), as reported (9, 15). Two complementary degenerate oligonucleotides (Genosys Biotechnologies, The Woodlands, TX) were first constructed for site-saturation mutagenesis at the Ser-130 position. For site-directed mutagenesis, specific oligonucleotides were designed based upon common codon usage (Genosys Biotechnologies).

**DNA Sequencing**—We performed DNA sequencing with an A.L.F. Express automated DNA sequencer (Amersham Biosciences) using the Thermo Sequenase™ fluorescent-labeled primer cycle sequencing kit in a manner similar to methods published previously (8, 9, 15).

**Antibiotic Susceptibility**—*E. coli* DH10B expressing the mutated *blat* genes were phenotypically characterized by LB agar dilution minimum inhibitory concentrations (MICs) using a “Steers replicator” that delivered 10⁴ colony forming units/spot. Antibiotics used and their suppliers were described previously (8, 9, 15). Concentrations employed for determining MIC values were in μg/ml. MICs were read after 18–24 h of incubation at 37 °C and performed three times for each antibiotic.

**Kinetics**—Kinetic constants of the SHV-1, S130G and S130T β-lactamases were determined by continuous assays at room temperature, 25 °C, using an Agilent® 8453 diode array spectrophotometer. Each experiment was performed in 20 mM phosphate-buffered saline at pH 7.4. Measurements were obtained using ampicillin (Δε₃₅₀ = 900 M⁻¹ cm⁻¹), piperacillin (Δε₈₀₂ = 5300 M⁻¹ cm⁻¹), cephalexin (Δε₇₆₀ = 7660 M⁻¹ cm⁻¹), and nitrocefin (Δε₄₉₂ = 17400 M⁻¹ cm⁻¹) (BD Biosciences). The kinetic experiments and analyses were patterned after the methods of Imtiaz *et al.* (17) and will be described briefly below.

The kinetic parameters, *Vₘₐₓ* and *Kₘₐₓ*, were obtained with non-linear least squares fit of the data (Michaelis-Menten equation) using EnzFitter™ (Sigma):

\[
V = \frac{V_{m,0} [S]}{K_{m,0} + [S]} 
\]

(1 Eq.)

A direct competition assay was performed to determine the dissociation constant for the preacylation complex, *Kᵟ*, of the inhibitors (clavulanic acid and tazobactam). We used a final concentration of 100 μM nitrocefin as the indicator substrate and 7 mM SHV-1 β-lactamase or 31 mM S130G β-lactamase in these determinations. The data were analyzed according to Equation 2 to account for the affinity of nitrocefin for the SHV-1 β-lactamase:

\[
K_{i} = \frac{K_{i,0}/1 + [S]/K_{i,0, nitrocefin}}{}
\]

(2 Eq.)

The first-order rate constant for enzyme and inhibitor complex being inactivated, *kₐₙₐₓ*, was measured directly by monitoring the reaction time courses in the presence of inactivators. A fixed concentration of inhibitor, nitrocefin, and increasing mM concentrations of inactivator, I, were used in each assay. The *kₐₙₐₓ* for inactivation was determined graphically as the reciprocal of the ordinate of the intersection of the straight lines obtained from the initial, *vᵢ*, and final, *vᵢₜ*, steady-state velocities. The *kₐₙₐₓ* was calculated from the relationship

\[
k_{a,n} = k_{a} [I]/(K_{i} + [I])
\]

(3 Eq.)

The second-order rate constant, *kₐₙₐₓ/Kᵟ*, the rate constant for reaction of free enzyme with free inhibitor to give inactive enzyme was determined by the fit of *kₐₙₐₓ* to Equation 3.

The partitioning of the initial enzyme inhibitor complex between hydrolysis and enzyme inactivation, *i.e.*, the turnover number (*tᵢ = kₐₙₐₓ/Kᵟ*) was obtained in the following manner. First, we incubated increasing amounts of inhibitor (clavulanic acid or tazobactam) with a fixed concentration of SHV-1 or S130G β-lactamase in a total volume of 100 μl of 20 mM phosphate-buffered saline, pH 7.4, at room temperature. After 24 h, an aliquot (10 μl) was removed from the mixture and analyzed for steady-state expression levels (16). The ELISA plate was read at λ = 492 nm and OD values of the samples were compared with an internal standard curve using the SHV-1 β-lactamase.

**β-Lactamase Purification**—SHV-1, S130G, and S130T β-lactamases were purified to homogeneity from *E. coli* DH10B cells according to a method employing preparative isoelectric focusing (8, 9, 15). We assessed the purity of each β-lactamase on 5% stacking and 12% resolving SDS-PAGE gels. Gels were stained with Coomassie Brilliant Blue R250 (Fisher) to visualize β-lactamases. Protein concentrations were determined with Protein Assay (Bio-Rad) using bovine serum albumin as a standard.

**Determination of Steady-state Expression Using an Enzyme-linked Immunosorbent Assay**—By using a polyclonal anti-SHV antibody, a quantitative enzyme linked immunosorbent assay (ELISA) method was employed to assess steady-state expression levels (16). The ELISA plates were read at λ = 492 nm and OD values of the samples were compared with an internal standard curve using the SHV-1 β-lactamase.

**ELISA**—Enzyme linked immunosorbent assay.

**Immunosorbent Assay**—By using a polyclonal anti-SHV antibody, a quantitative enzyme linked immunosorbent assay (ELISA) method was employed to assess steady-state expression levels (16).

**Kinetic parameters**

| Antibiotic | SHV-1 | S130G | S130T |
|------------|-------|-------|-------|
| Ampicillin | 16000 | 250   | 32    |
| Piperacillin | 2000 | 250   | ≤64   |
| Piperacillin/Clavulanic acid | 50/4 | 50/16 | 50/0.06 |
| Piperacillin/Tazobactam | 250/32 | 250/32 | 250/32 |

* Other amino acid substitutions.

**Table II**

| Kinetic parameters         | SHV-1 | S130G | S130T |
|----------------------------|-------|-------|-------|
| *kₐₙₐₓ*, μM⁻¹ s⁻¹       |       |       |       |
| Ampicillin                | 124 ± 7 | 290 ± 53 | ND   |
| Nitrocefin                | 5 ± 0.9 | 700 ± 88 | 88 ± 2 |
| Piperacillin              | 91 ± 10 | 89 ± 7  | 86 ± 1 |
| Cephaloridine             | 53 ± 10 | NM     | 73 ± 7 |

* ND, not determined; NM, not measurable.
a 90-min experiment. We reacted 1.6 μM SHV-1 and 25 μM S130G with 60 μM and 1 mM clavulanic acid, respectively, in a final volume of 200 μl. Similarly, we reacted 1.6 μM SHV-1 and 25 μM S130G with 7.75 μM and 125 μM of tazobactam, respectively. From these mixtures, a set aliquot was removed at designated time points, and steady-state velocities were measured.

The contributions of amino acid substitutions to the relative change in Gibbs free energy of the transition state with substrates and inhibitors were determined by using Equations 4 and 5 (18–20):

\[
\Delta G = -RT \ln(k_{\text{cat}}/K_m)_{\text{S130G}}/k_{\text{cat}}/K_m_{\text{SHV-1}} \quad \text{(Eq. 4)}
\]

\[
\Delta G = -RT \ln(k_{\text{max}}/K_{S130G}K_{\text{max}}/K_{S130G})_{\text{SHV-1}} \quad \text{(Eq. 5)}
\]

**Molecular Modeling**—Insight II™ software (Accelrys) was used to construct the S130G variant and to perform energy minimizations as described previously (9) based on the SHV-1 crystal structure (Protein Data Bank code 1SHV) (21).

**RESULTS**

**Mutagenesis**—Site-saturation and site-directed mutagenesis to obtain all 19 amino acid substitutions at the 130 position in SHV-1 was successfully performed, and the most common codons used were selected (8). The DNA sequence of each SHV was first determined in E. coli XL1 Blue cells.

**Antibiotic Susceptibility**—The effects of each amino acid substitution at the 130 position in the SHV β-lactamase were determined by using Equations 4 and 5 (18–20). For tazobactam, the difference in Kᵢ was also significantly elevated (Kᵢ = 0.07 μM for SHV-1 versus 4.2 μM for S130G; 60-fold increase). The difference in first-order rate constants for inactivation, k_{\text{inact}}/Kᵢ, was within 17% for clavulanic acid and 40% for tazobactam (Table IV). The second-order rate constants, k_{\text{inact}}/Kᵢ, demonstrate a larger difference between SHV-1 and S130G β-lactamase, the increase in Kᵢ being responsible for this.

In the time-dependent inactivation experiments, the ratio of I/E required to reduce activity by 90% (tₑ) was equal for SHV-1 and S130G at 24 h (clavulanic acid, 40/1; tazobactam, 5/1). At 20 min, the period corresponding to the dividing time of E. coli, the activity of S130G was reduced 55% by clavulanic acid; SHV-1 β-lactamase activity was reduced > 90% (Fig. 2). In contrast, by 15 min, SHV-1 and S130G β-lactamases were inactivated > 80% by tazobactam. The k_{\text{cat}} of both SHV-1 and S130G β-lactamases hydrolyze clavulanic acid and tazobactam (Table IV).

**Molecular Modeling**—Molecular modeling calculations indicate that the S130G β-lactamase is not significantly different in overall energy when compared with SHV-1, despite the loss of a key H-bonding residue in the active site. We do not observe other side chains close enough to assume the role of Ser-130 (22). In contrast, the catalytic water moves 2.37 Å nearer to the Lys-73:His22 atom. Additionally, there is movement of the important Lys-73 residue (0.5 Å for 73: H22) toward the catalytic Ser-70. The changes in active-site relationships as a result of the S130G substitution are shown in Fig. 3.

**DISCUSSION**

These data show that the S130G amino acid substitution is essential for resistance to ampicillin/clavulanic acid in SHV β-lactamase. The major effect of the S130G mutation on apparent Kᵢ and Kᵢ emphasizes the precise topological role of this amino acid (17, 22–27). A direct consequence of the Ser → Gly substitution is reflected by the amount of S130G versus SHV-1 inhibited in 20 min, the time required for E. coli cell division. The time-dependent inactivation of S130G by tazobactam occurred more rapidly than with clavulanic acid. The magnitude of the Kᵢ elevation combined with the rates of inactivation for tazobactam was not enough to result in an elevation of ampicillin/tazobactam (data not shown) or piperacillin/tazobactam MICs. This has been noted previously with laboratory isolates that produce larger amounts of β-lactamase than clinical strains.

In addition to these findings, two important observations of the kinetic behavior of SHV-1 and S130G β-lactamases with clavulanic acid and tazobactam deserve emphasis: S130G and SHV-1 β-lactamases are inactivated by clavulanic acid at sim-
Fig. 2. **Time-dependent inactivation experiments.** A, clavulanic acid inactivation of SHV-1 β-lactamase (♦) and S130G β-lactamase (■). B, tazobactam inactivation of SHV-1 β-lactamase (♦) and S130G β-lactamase (■).
**Fig. 3.** Stereo view of the superposition of the energy-minimized active-site structures of SHV-1 wild type (shown colored by element) and the S130G SHV variant (shown in magenta). Key active-site residues are highlighted as well as the catalytic water molecule (CAT H$_2$O). These structures were obtained using the AMBER force field.

**Fig. 4.** Proposed mechanism of inactivation of S130G β-lactamase with clavulanic acid (adapted from Refs. 10, 20, 21).
ilar rates, and hydrolysis rates are comparable. Analogous results were obtained with tazobactam. These data indicate that the S130G β-lactamase, as a clinically important β-lactam/β-lactamase inhibitor-resistant enzyme, maintains the fundamental hydrolytic mechanism common to all β-lactamases (12).

From our model (Fig. 3), we hypothesized that an active-site water molecule may relocate to a region nearer to the Lys-73 residue. The water molecule may also function with Lys-73 in a coordinated proton shuttle and may substitute for the Ser-130 bound carboxylic acid moiety would not be possible in the cross-linking or bridging species (vinyl ether), and the both the variant and wild-type enzymes. The absence of the inhibition observed in our experiments and would occur in trans process that preserves catalytic activity.

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