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The Role of a Second-Shell Residue in Modifying Substrate and Inhibitor Interactions in the SHV β -Lactamase: A Study of Ambler Position Asn276[†]

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Abstract

Inhibitor-resistant class A β -lactamases of the TEM and SHV families that arise by single amino acid substitutions are a significant threat to the efficacy of β -lactam/ β -lactamase inhibitor combinations. To better understand the basis of the inhibitor-resistant phenotype in SHV, we performed mutagenesis to examine the role of a second-shell residue, Asn276. Of the 19 variants expressed in *Escherichia coli*, only the Asn276Asp enzyme demonstrated reduced susceptibility to ampicillin/clavulanate (MIC increased from 50/2 \rightarrow 50/8 $\mu\text{g}/\text{mL}$) while maintaining high-level resistance to ampicillin (MIC = 8192 $\mu\text{g}/\text{mL}$). Steady-state kinetic analyses of Asn276Asp revealed slightly diminished $k_{\text{cat}}/K_{\text{m}}$ for all substrates tested. In contrast, we observed a 5-fold increase in K_{i} for clavulanate ($7.4 \pm 0.9 \mu\text{M}$ for Asn276Asp vs $1.4 \pm 0.2 \mu\text{M}$ for SHV-1) and a 40% reduction in $k_{\text{inact}}/K_{\text{i}}$ ($0.013 \pm 0.002 \mu\text{M}^{-1} \text{s}^{-1}$ for Asn276Asp vs $0.021 \pm 0.004 \mu\text{M}^{-1} \text{s}^{-1}$ for SHV-1). Timed electrospray ionization mass spectrometry of clavulanate-inhibited SHV-1 and

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SUPPORTING INFORMATION AVAILABLE MIC table of SHV-1 and Asn276 variants for cefotaxime and meropenem, tables of substrate and inhibitor efficiencies for a panel of IR SHV and TEM enzymes, immunoblot of Asn276 variants probed with SHV-1 polyclonal antibody, and proposed reaction intermediates observed in ESI-MS spectra of SHV-1 and SHV Asn276Asp inactivated with clavulanate are available free of charge via the Internet at <http://pubs.acs.org>.

SHV Asn276Asp showed nearly identical mass adducts, arguing for a similar pathway of inactivation. Molecular modeling shows that novel electrostatic interactions are formed between Arg244N η 2 and both 276AspO δ 1 and O δ 2; these new forces restrict the spatial position of Arg244, a residue important in the recognition of the C₃/C₄ carboxylate of β -lactam substrates and inhibitors. Testing the functional consequences of this interaction, we noted considerable free energy costs (+ G) for substrates and inhibitors. A rigid carbapenem (meropenem) was most affected by the Asn276Asp substitution (46-fold increase in K_i vs SHV-1). We conclude that residue 276 is an important second-shell residue in class A β -lactamase-mediated resistance to substrates and inhibitors, and only Asn is able to precisely modulate the conformational flexibility of Arg244 required for successful evolution in nature.

The development of β -lactam antibiotics (penicillins, cephalosporins, and carbapenems, Figure 1) remains one of the most significant advances in modern medicine. β -Lactam antibiotics manifest their bactericidal effects by inhibiting enzymes involved in cell wall synthesis. The broad spectrum of activity and low toxicity of β -lactams make them the “antimicrobial agents of choice” in the treatment of many infections. Unfortunately, the efficacy of these life-saving antibiotics is seriously threatened by the evolution of β -lactamases (1). β -Lactamases (E.C. 3.5.2.6) are periplasmic bacterial enzymes (hydrolases) that are frequently found in *Enterobacteriaceae* and are responsible for increasing resistance to penicillins, cephalosporins, and carbapenems. There are four classes of β -lactamases: classes A, C, and D possess Ser as the active site nucleophile, while class B uses a metal ion (Zn^{2+}) to inactivate β -lactams (2).

The most prevalent β -lactamases in *Enterobacteriaceae* (e.g., *Escherichia coli* and *Klebsiella pneumoniae*) are the class A enzymes (e.g., TEM and SHV)¹ (1). In order to overcome class A β -lactamase-mediated resistance and preserve the efficacy of these antibiotics, β -lactamase inhibitors (clavulanate, sulbactam, and tazobactam, Figure 1) were coupled with β -lactam antibiotics. These mechanism-based inhibitors greatly enhance the utility of their partner β -lactams (amoxicillin, ampicillin, piperacillin, cefoperazone, and ticarcillin) in the treatment of serious infections caused by Gram-negative bacteria in the hospital, skilled nursing facilities, and community settings (3).

Regrettably, selective pressure from excess antibiotic use accelerated the emergence of resistance to β -lactam/ β -lactamase inhibitor combinations. Single amino-acid substitutions in the TEM and SHV families of class A β -lactamases resulted in enzymes with reduced affinity for β -lactamase inhibitors (2, 4). At the time of this writing, 25 inhibitor-resistant TEMs (IRTs) and five inhibitor-resistant SHVs have been described (<http://www.lahey.org/studies/webt.asp>). “Inhibitor-resistant” refers to resistance to amoxicillin/clavulanate as defined by antimicrobial susceptibility testing and may, but does not necessarily, include resistance to sulbactam and tazobactam combinations (5). Because resistance to β -lactam/ β -lactamase inhibitor combinations challenges our ability to successfully treat serious

¹Abbreviations: TEM, class A β -lactamase named after the patient (Temoneira) from whom the first β -lactamase was isolated; SHV, class A β -lactamase named after the property sulfhydryl reagent variable; IR, inhibitor resistant; MIC, minimum inhibitory concentration; BATSI, boronic acid transition state inhibitor; IRT, inhibitor-resistant TEM; ESI-MS, electrospray ionization mass spectrometry; WT, wild-type; NCF, nitrocefin; PBS, phosphate-buffered saline; PDB, Protein Data Bank.

infections, attention has focused on designing and discovering novel inhibitors which can help overcome IR enzymes (6–9). A thorough understanding of the molecular basis of IR β -lactamases is essential to inform inhibitor development and anticipate future resistance determinants.

Currently, two primary structural changes explain resistance to clavulanate in TEM and SHV enzymes: (i) alterations in the geometry or shape of the “oxyanion hole” or electrophilic center; and (ii) changes in the position of Ser130 or Arg244 (we use the Ambler numbering system for class A β -lactamases) (10–15). The substitutions most frequently isolated in the clinic and producing the most pronounced inhibitor resistance in TEM include variants at Ambler positions Met69, Ser130, Arg244, Asn275, and Asn276 (16). Met69 and Ser130 substitutions are seen in clinical IR SHVs, but Arg244, Asn275, and Asn276 substitutions have not yet been detected. Despite 68% sequence identity between SHV and TEM enzymes, these closely related β -lactamases demonstrate differences in the kinetic details which confer resistance to clavulanate and sulbactam (13, 17).

Previous studies indicated that Asn276 in TEM-1 plays a critical role in inhibitor inactivation by way of its interaction with Arg244 (18, 19). Arg244 is positioned between the binding pocket and Asn276, and contributes directly to substrate and inhibitor affinity in TEM and SHV through hydrogen bonding with the conserved β -lactam carboxylate (5, 13, 20, 21). The Asn276Asp substitution in TEM results in an amoxicillin/clavulanate-resistant phenotype, reduced k_{inact} , and increased K_i values (18, 19, 22, 23). The X-ray crystal structure of TEM Asn276Asp highlighted the importance of the electrostatic environment surrounding residues 244 and 276 (19). The TEM Asn276Asp substitution created a new interaction with Arg244 (276AspO δ 2:Arg244N η 2); surprisingly, movement or repositioning of Arg244 was not observed. In the case of the wild-type (WT) TEM-1 enzyme crystal structure, there is only a hydrogen bond between Asn276 and Arg244; the TEM Asn276Asp variant has both a hydrogen bond and a salt bridge between these residues (24).

Based upon these reported findings, we were compelled to address the role of Asn276 in the SHV family of β -lactamases and discern its contribution to both substrate hydrolysis and clavulanate inhibition. Accounting for the different kinetic parameters of inhibition between TEM and SHV enzymes, we specifically inquired whether Asn276, located remotely from the active site (a second-shell residue), would have a major impact on the ability of SHV β -lactamase to discriminate between various β -lactams and clavulanate. To this end, we performed susceptibility testing on a full panel of amino acid variants at residue 276 constructed by site-saturation and site-directed mutagenesis and completed steady-state kinetic characterization of the clavulanate-resistant Asn276Asp variant. To compare the nature of the intermediates in the inactivation process, we inhibited SHV-1 and SHV Asn276Asp with clavulanate and resolved the resulting adducts by timed electrospray ionization mass spectrometry (ESI-MS). In addition, novel boronic acid transition state inhibitors (BATSIs), a methylidene penem, and meropenem (Figure 1) were tested as probes of the functional consequences of the Asn276Asp substitution on β -lactam carboxylate recognition and affinity. Our data leads us to conclude that in both SHV and TEM, perturbations of the electrostatic relationships between Asp276 and Arg244 create unique structural/conformational changes that have significant consequences on the turnover of

substrates and inhibitors. However, the Asn276Asp substitution in SHV “enjoys” clavulanate resistance at a lower cost to catalytic efficiencies than most other IR SHVs and IRTs.

MATERIALS AND METHODS

Mutagenesis and Sequencing

Using the template *bla*_{SHV-1} gene in the phagemid vector pBC SK(-) (Stratagene, La Jolla, CA), we employed Stratagene’s QuikChange Mutagenesis kit with degenerate oligonucleotides at Ambler position 276 to make the full complement of amino acid substitutions. After PCR mutagenesis, we electroporated the resultant plasmids into *E. coli* ElectroMAX DH10B cells (Invitrogen, Carlsbad, CA). These plasmids were isolated and DNA sequencing of the *bla*_{SHV} genes was performed. Variants not acquired after sequencing of 100 clones were created with specific mutagenic oligonucleotides as previously described (25).

Immunoblotting

E. coli DH10B cells were grown to OD₆₀₀ = 0.8 and lysed by 10 min incubation at 100 °C in SDS loading dye buffer. Immunoblots were performed with an anti-SHV polyclonal antibody to confirm production of the SHV β-lactamases from all 19 constructs as previously described (26).

Antimicrobial Susceptibility Tests

MICs for *E. coli* DH10B cells expressing the *bla*_{SHV-1} or mutant *bla*_{SHV Asn276Xxx} genes cloned in phagemid pBC SK(-) were obtained by lysogeny broth agar dilution. The MICs for various antibiotics were determined using a Steers Replicator that delivered 10 μL of a diluted overnight culture containing 10⁴ colony forming units. Ampicillin, piperacillin, cephalothin, and cefotaxime were purchased from Sigma (St. Louis, MO); meropenem was purchased from AstraZeneca Pharmaceuticals (Wilmington, DE). Lithium clavulanate (GlaxoSmithKline, Surrey, United Kingdom), sulbactam (Pfizer, La Jolla, CA), and tazobactam (Wyeth Pharmaceuticals, Pearl River, NY) were kind gifts and were tested in combination with 50 μg/mL ampicillin.

β-Lactamase Expression and Purification

β-Lactamase enzymes were expressed and purified as described previously (27). Briefly, *E. coli* DH10B cells containing the *bla*_{SHV-1} or *bla*_{SHV Asn276Asp} genes in pBC SK(-) were grown overnight in SOB medium, harvested by centrifugation at 4 °C, and frozen at -20 °C. After thawing, β-lactamase was liberated using stringent periplasmic fractionation with 40 μg/mL lysozyme (Sigma) and 1 mM EDTA, pH 7.8. Preparative isoelectric focusing was performed with the lysate in a Sephadex granulated gel (GE Healthcare, Piscataway, NJ) using ampholines in the pH range 3.5–10 and running the gel overnight at a constant power of 8 W on a Multiphor II isoelectric focusing apparatus (GE Healthcare). Purity was assessed by 5% stacking, 12% resolving sodium dodecyl sulfate–polyacrylamide gel electrophoresis. β-Lactamase concentration was determined using the Bio-Rad (Hercules, CA) Bradford protein assay with bovine serum albumin standards. If purity was <90%, a

second purification step was performed using size-exclusion chromatography with a Pharmacia ÄKTA Purifier system (fast protein liquid chromatography instrument) (Uppsala, Sweden) (28).

Kinetic Measurements

Steady-state kinetics were performed using continuous assays at room temperature in an Agilent 8453 diode array spectrophotometer (Agilent, Palo Alto, CA). Each assay was performed in 10 mM phosphate-buffered saline (PBS) at pH 7.4. Measurements were obtained using nitrocefin (NCF) (BD Biosciences, San Jose, CA) ($\epsilon_{482} = 17400 \text{ M}^{-1} \text{ cm}^{-1}$), ampicillin ($\epsilon_{235} = -900 \text{ M}^{-1} \text{ cm}^{-1}$), piperacillin ($\epsilon_{235} = -820 \text{ M}^{-1} \text{ cm}^{-1}$), and cephalothin ($\epsilon_{262} = -7660 \text{ M}^{-1} \text{ cm}^{-1}$). In order to specifically investigate the role of the C₃/C₄ carboxylate in substrate recognition, we used (i) an achiral and chiral cephalothin BATSI (13), (ii) a methylidene penem inhibitor bearing a dihydropyrazolo[1,5-c][1,3]thiazole moiety (Wyeth Pharmaceuticals) (29), and (iii) the carbapenem meropenem.

The kinetic parameters, V_{\max} and K_m , were obtained with nonlinear least-squares fit of the data (Henri Michaelis–Menten equation) using Origin 7.5 (OriginLab, Northampton, MA)

$$v = (V_{\max} * [S]) / (K_m + [S]) \quad (1)$$

The dissociation constants of the preacylation complex, K_i s, were determined by direct competition assays. The initial velocity was measured in the presence of a constant concentration of enzyme (10 nM) with increasing concentrations of inhibitor against the indicator substrate, NCF. Care was taken to initiate the reaction with the addition of both NCF and the inhibitor and to limit the initial velocity determination to the first 5 s.

K_i values for the BATSIs were determined as described above. However, because of an incubation effect, SHV β -lactamase and the chiral boronic acid compound were preincubated for 5 min in PBS before initiating the reaction with the addition of substrate (NCF), as has been described previously (9, 13).

The first-order rate constant for enzyme and inhibitor complex inactivation, k_{inact} , was obtained by monitoring the reaction time courses in the presence of inhibitor. Fixed concentrations of enzyme (10 nM) and NCF (150 μM) and increasing concentrations of inhibitor were used in each assay. The k_{obs} was determined using a nonlinear least-squares fit of the data using Origin 7.5:

$$A = A_0 + v_f t + (v_0 - v_f) [1 - \exp(-k_{\text{obs}} t)] / k_{\text{obs}} \quad (2)$$

Here, A is absorbance, v_0 (expressed in variation of absorbance per unit time) is initial velocity, v_f is final velocity, and t is time. Each k_{obs} was plotted versus I and fit to determine k_{inact} . The value of k_{inact} was used to determine K_I (equivalent to K_m) according to the following equation using Origin 7.5:

$$k_{\text{obs}} = (k_{\text{inact}} [I]) / (K_I + [I]) \quad (3)$$

Both the K_i and K_I data were corrected to account for the affinity of NCF for SHV-1 and Asn276Asp according to the following equations (30):

$$K_i (\text{corrected}) = K_i (\text{observed}) / [1 + ([S]/K_m \text{NCF})] \quad (4a)$$

$$K_I (\text{corrected}) = K_I (\text{observed}) / [1 + ([S]/K_m \text{NCF})] \quad (4b)$$

The partitioning of the initial enzyme inhibitor complex between hydrolysis and inactivation, that is, the partition ratio ($k_{\text{cat}}/k_{\text{inact}}$ or t_n), was calculated by plotting the relative β -lactamase activity against the inhibitor/enzyme ratio as previously described by Bush et al. (31). Briefly, increasing amounts of inhibitor were incubated with a fixed concentration of β -lactamase (10 nM). After 24 h, an aliquot was removed from the mixture and the initial velocity was measured and compared with a control sample with no inhibitor added. The proportion of inhibitor to β -lactamase that resulted in 90% inactivation after 24 h was defined here as the partition ratio.

We calculated the difference in binding energies between SHV-1 and Asn276Asp β -lactamases and the substrates and inhibitors (32, 33). For the substrates, the interaction energies of the acyl-enzyme of SHV-1 relative to Asn276Asp (ΔG) were determined from the following equation where R is the universal gas constant and T is in degrees Kelvin (33):

$$\begin{aligned} \Delta\Delta G &= \Delta G (\text{SHV} - 1) - \Delta G (\text{Asn276Asp}) \\ &= -RT \ln \left[(K_m \text{SHV-1}) / (K_m \text{Asn276Asp}) \right] \quad (5) \end{aligned}$$

For the inhibitors, K_i values were used to obtain the differential energies needed to form the preacylation complex with SHV-1 or Asn276Asp.

Mass Spectrometry

For intact protein mass spectrometry to determine intermediates and products of inactivation, we incubated 40 μM of SHV-1 or Asn276Asp with and without addition of 40 mM clavulanate for 15 min. Each reaction was terminated by the addition of 0.1% trifluoroacetic acid and immediately desalted and concentrated using a C₁₈ ZipTip (Millipore, Bedford, MA) according to the manufacturer's protocol. Samples were then placed on ice and analyzed within 1 h.

Spectra of the intact SHV-1 and Asn276Asp proteins were generated on an Q-STAR XL quadrupole-time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a nanospray source. Experiments were performed by diluting the protein sample with 50% acetonitrile/0.1% trifluoroacetic acid to a concentration of 10 μM . This protein solution was then infused at a rate of 0.5 $\mu\text{L}/\text{min}$ and the data were collected for 2 min. Spectra were deconvoluted using the Analyst program (Applied Biosystems). All measurements have an error of ± 3 Da.

Molecular Modeling

The crystal structure coordinates of the SHV-1 β -lactamase from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB entry 1SHV) were used to generate a representation of SHV Asn276Asp β -lactamase inactivated by clavulanate. Hydrogen atoms were added (pH = 7.5), and the structure minimized at a constant dielectric of 1 with the conjugate gradient method and a Constant Valence Force Field (Accelrys Insight II, Discover_3, San Diego, CA). The energy minimization of the PDB file was followed by an Asn276Asp substitution. The model for clavulanate was constructed using the Builder Module of Insight II. In order to represent the acyl-enzyme intermediate, a bond was created between C₇ of clavulanate and Ser70. The enzyme-inhibitor complex was energy minimized to a convergence of 0.02Å (10 000 iterations).

RESULTS

Construction of Variants at Ambler Position 276; Mutagenesis and Immunoblotting

Using degenerate oligonucleotides, we performed site-saturation mutagenesis on the *bla*_{SHV-1} gene at Ambler position 276. Seventeen of 19 amino acid substitutions were obtained in the initial sequencing screen (100 *bla*_{SHV} genes selected and sequenced). *bla*_{SHV Asn276Phe} and *bla*_{SHV Asn276Thr} were constructed by site-directed mutagenesis. Expression of β -lactamase proteins was confirmed by immunoblotting. With the possible exception of the SHV Asn276Pro variant, all enzymes were expressed at steady state (0.8 OD) (Supporting Information, Figure 1).

Antimicrobial Susceptibility Tests

To test the impact of the single amino acid substitutions at Asn276 on β -lactam susceptibility, MICs against all 19 variants were determined in a uniform *E. coli* DH10B background. Results are summarized in Table 1.

All Asn276 variants possessed lower MICs for the penicillins and cephalothin as compared to SHV-1. The ampicillin and piperacillin MICs (8192 and 512 μ g/mL, respectively) were relatively preserved for the Asn276Asp variant compared to the remaining Asn276 variants, suggesting that the Asn276Asp variant retains greater ability to hydrolyze penicillins. It is particularly noteworthy that there is a very wide range of MICs (2–8192 μ g/mL) and that the pattern seen is not based upon size of the R group, charge, polarity, or hydrophobicity. Each of the variants at Asn276 demonstrated reduced susceptibility to cephalothin (range 4–16 μ g/mL). MICs against the extended-spectrum cephalosporin cefotaxime and meropenem were also performed and all Asn276 variants had values comparable to SHV-1 (Supporting Information, Table 1).

With regard to β -lactam/ β -lactamase inhibitor combinations, only the Asn276Asp enzyme resulted in a higher MIC value for the ampicillin/inhibitor combinations: increased resistance to ampicillin/clavulanate and a value comparable to SHV-1 for ampicillin/sulbactam and ampicillin/tazobactam.

Kinetic Behavior of Asn276Asp with β -Lactam Substrates

Because of its phenotype and potential clinical relevance, we purified the Asn276Asp variant enzyme for kinetic analysis. Except for ampicillin, we observed that the Asn276Asp variant has a higher K_m against all substrates (Table 2). In contrast, the k_{cat} value is lowered save for piperacillin, which has a value similar to SHV-1. Overall, the catalytic efficiencies, k_{cat}/K_m , were slightly reduced for the Asn276Asp variant.

Kinetic Behavior of Asn276Asp with Clavulanate

Table 3 summarizes the kinetic data for SHV-1 and the Asn276Asp variant inactivated by clavulanate. Both the clavulanate K_i and K_I values are increased for Asn276Asp as compared to SHV-1 (5.4- and 1.6-fold, respectively). Unlike studies of SHV Arg244Ser, we did not find a difference in clavulanate k_{inact} for Asn276Asp and SHV-1 (13). We measured the partition ratio for both SHV-1 and Asn276Asp and observed only a slight increase in the number of clavulanate molecules hydrolyzed by the variant enzyme before inactivation was achieved (40 vs 50). Similarly, the k_{cat} calculations indicate a small increase in the rate of inhibitor hydrolysis for the Asn276Asp variant.

Kinetic Behavior of Asn276Asp with β -Lactamase Inhibitors: Probes of the Active Site

In order to investigate the functional interactions responsible for the SHV Asn276Asp IR phenotype, we assayed two BATSIs, an investigational methylidene penem, and meropenem for their activities against SHV-1 and Asn276Asp enzymes (Table 3). The BATSI tested contain the R_1 side chain of cephalothin and differ only by the addition of a *meta*-carboxyphenyl group on the chiral compound. The *meta*-carboxyphenyl moiety contains a carboxylate that is linked to the sp^2 -hybridized carbon atom of a phenyl ring, and is intended to imitate the interactions with the C_3/C_4 carboxylate of penicillins and cephalosporins. These boronic acid compounds allow us to assess the extent to which the Asn276Asp substitution contributes to affinity for the substrate and/or inhibitor carboxylate by its interaction with Arg244 (13).

The chiral BATSI with the *meta*-carboxyphenyl group demonstrated 62-fold greater affinity for SHV-1 than the achiral compound (42 μ M vs 0.68 μ M, respectively), suggesting that interactions with the C_3/C_4 carboxylate are very important for increasing affinity for the enzyme's active site. Testing the BATSI with Asn276Asp also showed that the presence of the *meta*-carboxyphenyl on the chiral compound increased affinity, but the improvement in K_i of binding the chiral compound over the achiral was reduced to only 9-fold (36 μ M vs 3.8 μ M, respectively).

We next studied a methylidene penem that contains an sp^2 -hybridized C_3 bearing the carboxylate and a bicyclic R_1 side chain. The K_i and K_I values of the penem for SHV-1 and SHV Asn276Asp were in the nM range, with modest increases in both constants for the Asn276Asp variant (1.7- and 1.5-fold for K_i and K_I , respectively). The k_{inact} rate was also slightly higher for the Asn276Asp variant, but the overall inhibitor efficiency (k_{inact}/K_I) and partition ratios were very similar between SHV-1 and Asn276Asp enzymes.

Meropenem resists hydrolysis by SHV β -lactamases and forms a stable acyl-enzyme (34). Here, the affinity of meropenem for Asn276Asp was assessed by a competition reaction with NCF to obtain the dissociation constant, K_i . The affinity loss of the Asn276Asp enzyme for the carbapenem was greatest, a 46-fold increased K_i as compared to SHV-1.

In Table 4, we summarize our calculations of differences in binding energies between SHV-1 and Asn276Asp (see eq 5). With the exceptions of ampicillin and the achiral cephalothin BATS1, where energy differences are very small, the Asn276Asp enzyme required more energy to bind substrates and inhibitors than did SHV-1.

Determining the Nature of the Intermediates: ESI-MS of SHV-1 and Asn276Asp with Clavulanate

Timed ESI-MS was performed with SHV-1 and Asn276Asp β -lactamase inactivated by clavulanate using an inhibitor/enzyme ratio of 1000:1 to ensure detection of the covalent intermediates in the inactivation pathway. As shown in Figure 2, when SHV-1 and Asn276Asp were incubated with clavulanate for 15 min, nearly identical covalent intermediates were formed. This included adducts at +51, +70, +88, +140, and +158 which were observed previously with TEM-2, SHV-1, and the IR SHV Arg244Ser (all measurements have an error of ± 3 Da, see Supporting Information, Figure 2, for proposed reaction intermediates corresponding to adducts) (13, 35, 36). These similarities in the spectra of inactivation species argue strongly for a common pathway and reaction mechanism.

DISCUSSION

We undertook an in-depth study of Ambler position 276 in SHV to enhance our understanding of the IR phenotype in class A β -lactamase enzymes, and to explore potentially relevant differences between IR SHVs and IRTs. Our goal was to elucidate the role of this second-shell residue on the catalytic profile of SHV enzymes.

The Impact of Substitutions at Ambler Position Asn276 on Phenotype

Susceptibility testing of the full repertoire of SHV Asn276 variants showed that only the Asn276Asp enzyme was uniquely able to reduce susceptibility to ampicillin/clavulanate. Furthermore, this variant displayed another distinct feature that contrasts what is typically observed with IR enzymes: the Asn276Asp variant retained a high level of resistance to ampicillin and piperacillin (Table 1). It is noteworthy that all the other variants at position 276 were uniformly less resistant to ampicillin/sulbactam and ampicillin/tazobactam than SHV-1 expressed in *E. coli* DH10B. This pattern was observed by Thomson et al. in studies exploring the role of Arg244 in SHV (5, 13). On the basis of the Asn276Asp enzyme's ability to balance these "desirable" catalytic properties (i.e., avoiding inhibition by clavulanate and retaining hydrolytic activity for penicillins) and its potential clinical relevance, we selected Asn276Asp for detailed study.

Steady-State Kinetics

Steady-state kinetic analyses were performed to explain the biochemical correlates of the Asn276Asp phenotype for substrate and inhibitor catalysis. Overall, we found that the Asn276Asp β -lactamase is moderately impaired when compared to SHV-1 (k_{cat}/K_m ratios are 41–75% of WT) (Table 2). Of the parameters measured, the K_m and catalytic efficiency (k_{cat}/K_m) for the penicillins were least affected by the 276 substitution. NCF and cephalothin demonstrated slightly greater reductions in affinities (higher K_m) and turnover (lower k_{cat}). The most striking difference compared to SHV-1 was that Asn276Asp showed a 46-fold change in K_i for meropenem. Based upon previous analysis and work in SHV performed by Nukaga et al. and Thomson et al., we attribute this pattern (loss of penicillin > cephalothin > meropenem affinities) to the interaction of the Asn276Asp β -lactamase with the C₃/C₄ carboxylate of these substrates (13, 34). Carboxylates linked to C₃ or C₄ sp^2 -hybridized carbons (meropenem and cephalothin, respectively) have reduced rotational freedom due to conjugation as compared to carboxylates linked to a C₃ sp^3 -hybridized carbon (i.e., penicillins). Thomson et al. suggested that the carboxylate linked to a C₃/C₄ sp^2 -configured carbon is brought in closer approximation to Arg244 of SHV in the Henri-Michaelis complex, likely leading to increased hydrogen bonds with this residue (13). This contrasts with the notion that Arg244 may make only one relatively weak hydrogen bond with the penicillin carboxylate linked to a C₃ sp^3 -hybridized carbon; the anchoring interactions come from other residues in the binding pocket, such as Thr235 (20). Thus, by examining substrates with different stereochemistry at the C₃/C₄ position, we infer that the substitutions at residue 276 in SHV affect the affinity and kinetics of how Arg244 binds to substrates.

Resistance to clavulanate in SHV Asn276Asp is mediated by decreased affinity. This is a property shared with the IR TEM Asn276Asp enzyme. However, the magnitude of affinity loss is less for the SHV variant than the TEM variant (18, 19). The inactivation efficiencies (k_{inact}/K_I) reveal that SHV Asn276Asp retains more susceptibility to clavulanate (19).

Molecular Modeling

On the basis of phenotypic and kinetic study of SHV Asn276Asp, we created a molecular model to complement our observations. Our model reveals that new electrostatic interactions are created by the Asn276Asp substitution in SHV (Figure 3). In SHV Asn276Asp, the distances between atoms N η 2 of Arg244 and both O δ 1 and O δ 2 of 276Asp are decreased as compared to SHV-1, by 0.9 and 0.6 Å, respectively. These observations are reminiscent of the distances observed in the atomic structure of TEM Asn276Asp (N η 2 of Arg244 and O δ 2 of 276Asp are closer by 1.0Å) (19, 24). Yet, why does Asn276Asp maintain catalytic efficiency against penicillins, demonstrate resistance to inactivation by clavulanate, and show such wide changes in K_i when probes are tested? Why are all the other 276 substitutions so ineffective? We posit that the Asp substitution is singular in its ability to create a more “rigid enzyme” by impairing the conformational flexibility of Arg244. Close inspection of the Ne atom of Arg244 in the crystal structures of TEM-1 and TEM Asn276Asp (PDB entries 1BTL and 1CK3, respectively) reveals that this residue exists in multiple conformations. The two new interactions between Arg244 and 276Asp in SHV Asn276Asp restrict facile hydrogen bond formation of the Arg244 guanidinium group with the C₃ carboxylate of clavulanate. As Arg244 plays a key role in substrate recognition, we

would anticipate that major effects would be seen for substrates as well. Our MIC data show that 18 of the 19 substitutions at Asn276 result in a less robust penicillinase as a result of this rigidity (the other residues at 276 do not permit productive interactions with Arg244). Most importantly, we also see resistance to clavulanate. This latter property is most likely a consequence or benefit of restricted flexibility. This argument is supported by the increased K_m , K_i , and G values and changes in MICs.

To explore further the functional interactions of the Asn276Asp substitution, we next turned to inhibitors (BATSI, a methylidene penem, and meropenem) with carboxylates linked to sp^2 -hybridized atoms. The inhibitors were chosen because they help us answer (i) the extent to which the position and stereochemistry of the carboxylate impacts affinity for the Asn276Asp variant and (ii) if other interactions besides Arg244-Asn276Asp are important for inhibitor binding.

BATSI were selected because they replace the β -lactam motif with boronic acid and form reversible, dative covalent bonds with the active site serine (37, 38). This leads to formation of an adduct which resembles the geometry of the tetrahedral transition state of the β -lactamase hydrolytic reaction. By modifying these compounds to contain the R_1 side chains of natural substrates at the same distance from the electrophilic moiety, affinities in the nanomolar range for both class A TEM-1 and extended-spectrum-type SHV and class C enzymes were reported (39, 40). Here, we tested one achiral BATSI bearing the R_1 amide side chain found in cephalothin and a second chiral BATSI that has an additional *meta*-carboxyphenyl ring on the carbon atom alpha to the boron which resembles the dihydrothiazine ring of cephalosporins. Since the geometry and the distances in the chiral BATSI are the same as in the natural substrate, the carboxylate moiety of the *meta*-carboxyphenyl group corresponds to the C_3/C_4 carboxylate found on all β -lactams. Thus, comparing affinity of the achiral and chiral compounds allowed us to probe selectively how the Asn276Asp substitution affects binding of the carboxylate of cephalosporins.

The affinity gain of the carboxylate (i.e., the difference in K_i between the achiral and chiral compounds) for the SHV-1 enzyme was almost seven times increased for the Asn276Asp variant. Because of the selective design of these inhibitors, we attribute this difference to a less favorable interaction between Arg244 and the inhibitor carboxylate in the Asn276Asp variant. Quantitatively, the *meta*-carboxyphenyl group contributes 1.0 kcal/mol less energy to the affinity of the enzyme interaction in the Asn276Asp variant as compared to SHV-1 (Table 4). In total, these results support our hypothesis that residue 276, despite not making direct bonds with the substrate, plays a significant role in coordinating the carboxylate in the substrate-enzyme complex.

Methylidene penems and their derivatives are effective inactivators of classes A, C, and D β -lactamases, including IR class A enzymes (5, 41–43). Assays of one of these novel penems yielded similar inhibitor efficiencies for SHV-1 and Asn276Asp. As proposed in studies with the SHV Arg244Ser variant, the affinity losses at the C_3 carboxylate of the penem may be compensated by favorable interactions with the inhibitor's bicyclic R_1 side chain (5). Previous molecular models and structure determinations have shown that the R_1 side chain may be involved in π - π interactions with Tyr105 (42). Despite the sp^2 -

hybridized carboxylate, the preserved efficiency of this penem against the clavulanate-resistant Asn276Asp variant stresses that more than one significant (compensatory) interaction is possible in the active site (43).

In contrast to stabilizing interactions of the R₁ side chain of methyldene penem inhibitors, carbapenems contain a hydroxyethyl group at the R₁ position and a large R₂ side chain, both of which have destabilizing effects in crystal structures of carbapenem/class A β -lactamase complexes (34, 44, 45). Thus, that SHV Asn276Asp experiences the greatest affinity loss for meropenem, as compared to all other substrates and inhibitors tested, is consistent with the notion that the interactions between the variant enzyme and the ligand carboxylate are impaired. Meropenem, with its limited flexibility and rigid carboxylate on the *sp*² carbon atom, requires an additional 2.3 kcal/mol for formation of the preacylation complex with Asn276Asp vs SHV-1 (Table 4). Previous studies of the SHV Arg244Ser variant revealed a cost of 3.6 kcal/mol for the same process; perturbations of the inherent flexibility of residue 244 impair carboxylate recognition (13).

Comparison to IR SHV and TEM Enzymes

Can we compare IR SHV enzymes with IRTs and what do we learn from this? Examined together, our analysis of substrate and inhibitor catalysis in SHV-1 and SHV Asn276Asp represents a “fine tuning” of the IR phenotype. In general, IR enzymes must establish a balance between retaining the ability to hydrolyze β -lactam substrates, but decreasing efficacy of the structurally related β -lactam inhibitors (4). The majority of the IR enzymes “accept” a significant decrease in substrate hydrolytic efficiency in exchange for dramatically decreased inhibitory efficiency. This produces an IR phenotype when organisms expressing these enzymes are tested against β -lactam/ β -lactamase inhibitor combinations. MICs represent the “sum” of an enzyme’s ability to hydrolyze the combination of both β -lactam (e.g., ampicillin) and the β -lactamase inhibitor (e.g., clavulanate) in a relatively short time frame. Our data shows that in the case of SHV Asn276Asp, the increased ampicillin/clavulanate resistance results from a small decrease in clavulanate inhibition, and is also due to the preserved ability of the enzyme to hydrolyze the ampicillin present in the β -lactam/ β -lactamase inhibitor combination. We further illustrate these unique properties of SHV Asn276Asp by comparison with the kinetic data of a collection of IR SHV and IRT variants; first, the relatively high catalytic efficiency for ampicillin; and second, the slightly decreased inhibitory efficiency of clavulanate (see Supporting Information, Tables 2 and 3, for substrate and inhibitor efficiencies of a panel of IR SHV and IRT enzymes). The Asn276Asp alteration of the IR phenotype in the SHV β -lactamase may represent a significant evolutionary advance, as the enzyme maintains a balance of the desired catalytic properties.

For clavulanate-resistant TEM enzymes, substitutions at Asn276 and Arg244 lead to decreased occupancy of a water molecule essential for inactivation by clavulanate (18–20, 46). To support our model for SHV, we propose that the alteration imposed by the Asn276Asp substitution may not affect the (yet to be identified) water molecule crucial to clavulanate inactivation in SHV (Figure 4). Previous examination of SHV and TEM crystal structures suggests that SHV enzymes may not rely on the water molecule coordinated by

Arg244 and Val216 for inactivation by clavulanate (13, 17, 24). Consequently, the attenuated IR SHV Asn276Asp phenotype is produced by decreased affinity for the carboxylate of the inhibitors, and not by disruption of the inactivation mechanism from a dislocated water molecule. These important differences in TEM and SHV mechanisms support a divergent evolution of these enzymes viz a viz substrate and inhibitor profiles.

Thus, we posit that the SHV Asn276Asp variant may exist in clinical isolates of *Enterobacteriaceae*. However, as a result of the attenuated IR phenotype, this variant has not risen to clinical attention. This is in contrast to the TEM Asn276Asp enzyme, which exhibits a more significant decrease in catalytic activity, but more pronounced IR. In general, the prevalence of IR β -lactamases may be underestimated as identification of IR enzymes requires specific kinetic characterization and susceptibility testing (4, 47). Recognition of existing IR SHV variants, particularly those with a less pronounced phenotype, may have escaped detection.

Conclusion

In summary, we highlight the important role of a second-shell residue in class A β -lactamases and describe the unique properties of Ambler residue Asn276 in SHV. Structure–function studies of proteins often focus on first-shell residues that make direct interactions with active-site ligands. Recent studies of metallo-enzymes, including metallo- β -lactamases, have emphasized that second-shell residues can contribute to metal binding affinities and active site charge distributions (48, 49). Additional investigations show that second- and third-shell residues in nitric oxide synthase enzymes modulate important conformational changes in invariant first-shell residues (50). In these proteins, new binding pockets are revealed upon ligand binding, and selective inhibitors can be rationally designed to take advantage of how the second-shell residues modulate enzyme flexibility. Our data demonstrate that perturbations of the β -lactamase second-shell residue 276 are transferred to the active site by interaction with Arg244 and manifested in decreased affinity for piperacillin, NCF, cephalothin, and clavulanate. This new interpretation and deeper understanding for the tertiary structure and interactions between residues are essential to the success of selective and effective inhibitors of these β -lactamase enzymes. The lessons learned from the “finely tuned” enzyme can focus our efforts to meet the challenges raised by the impressive efficiency of these β -lactamases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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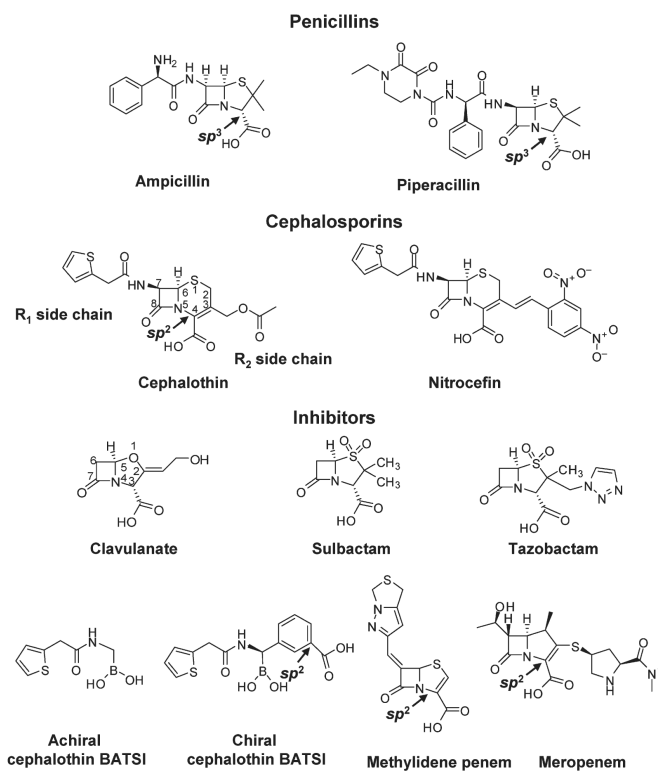
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**FIGURE 1.**

Chemical structures of compounds tested in this study. The structures of cephalothin and clavulanate are labeled with the accepted ring numbering system.

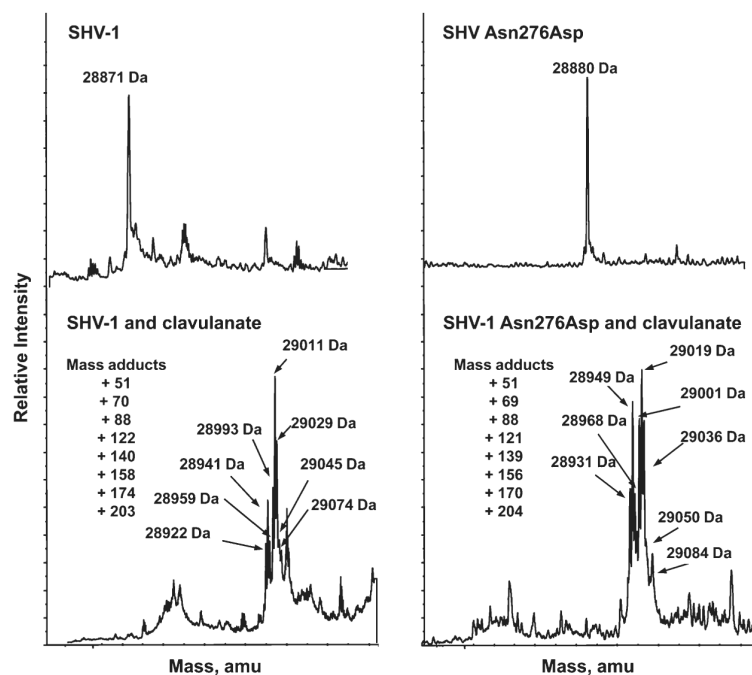


FIGURE 2.

Deconvoluted ESI-MS spectra of (A) SHV-1 and (B) SHV Asn276Asp before and after 15 min inactivation with clavulanate at a inhibitor/enzyme ratio of 1000:1. All measurements have an error of ± 3 Da. Eight distinct mass shifts were identified with both enzymes inactivated with clavulanate. This includes adducts postulated to represent: + 51, terminally inactivated cross-linked or propynoyl enzyme species; + 70, aldehyde; + 88, hydrated aldehyde; + 158, decarboxylated imine; and + 198, acyl-enzyme, imine, cis- or *trans*-enamine (see Supporting Information, Figure 2 for structures of proposed reaction intermediates) (13, 35).

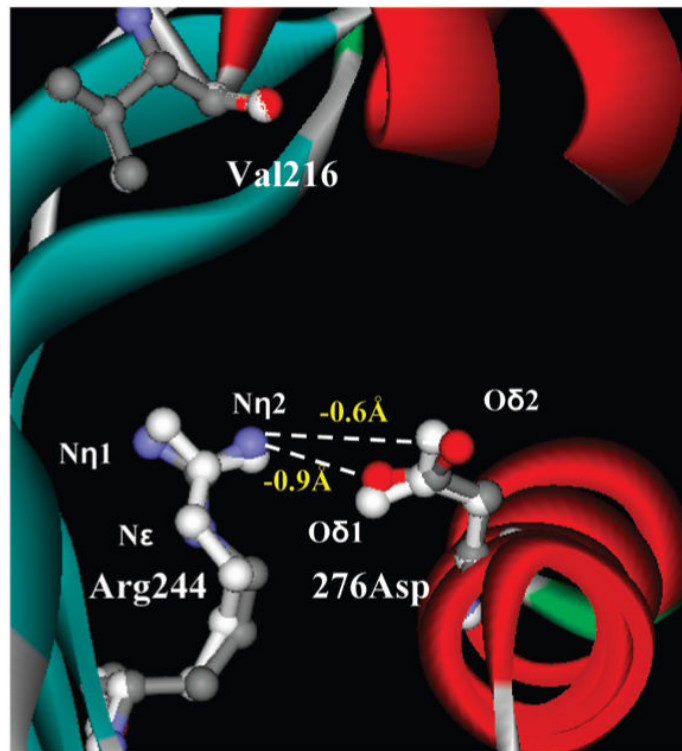
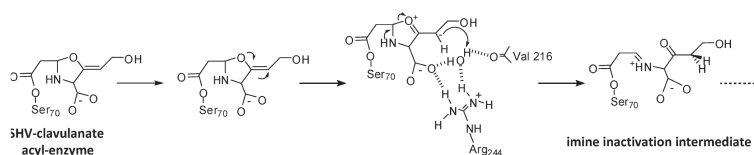


FIGURE 3. Molecular representation of SHV Asn276Asp based on SHV-1 (PDB entry 1SHV) showing the increased interaction between Arg244 and 276Asp mediated by shortened distances between Arg244 N η 2 and both 276Asp O δ 1 and O δ 2 (decreased by 0.9 and 0.6 Å, respectively). Residue 216 is shown for reference. SHV-1 amino acid positions in white and SHV Asn276Asp model in color.

**FIGURE 4.**

Proposed reaction mechanism for SHV-1 and clavulanate showing the formation of the imine inactivation intermediate. Scheme shows the contribution of Arg244 in the coordination of the water molecule hypothesized to donate a proton for C₂ double bond saturation and subsequent secondary ring-opening. In SHV-1, it is postulated that the water molecule is recruited or relocated into the active site with the binding of clavulanate (13). The crystal structure of TEM Asn276Asp (PDB entry 1CK3) reveals that this crucial water is missing, offering an explanation for the IR phenotype as protonation of clavulanate would be impaired, and thus lead to more ready hydrolysis of the inhibitor than in TEM-1, where the water is clearly refined (19).

Table 1MIC values ($\mu\text{g/mL}$) of *E. coli* DH10B Expressing SHV-1 and Asn276 Variants^a

	ampicillin	piperacillin	cephalothin	ampicillin/clavulanate	ampicillin/sulbactam	ampicillin/tazobactam
DH10B	1	1	4	0.06	0.06	0.06
SHV-1	> 16384	2048	64–128	2	512	64
Asn276Asp	8192	512	8–16	8	256	64
Asn276Gly	4096	256	16	2	64	16
Asn276Ser	4096	512	16	1	32–64	16
Asn276Val	2048	512	16	0.5	256	32
Asn276Thr	1024–2048	128–256	8–16	1	32	8
Asn276Gln, -Glu, -Cys	1024–2048	64–128	8–16	0.5	64–128	8
Asn276His	1024	256	16	1	128	16
Asn276Ala, -Leu, -Ile	256–512	16–32	4–8	0.25–0.5	8–16	2
Asn276Lys, -Arg, -Tyr, -Phe	64	8–16	4–8	0.125–0.25	1–4	0.5–1
Asn276Met	32	4	4	0.06	0.06	0.06
Asn276Pro, -Trp	8–16	2–4	4–8	0.06	0.06	0.06

^aInhibitors were evaluated in the presence of 50 $\mu\text{g/mL}$ ampicillin.

Table 2

Kinetic Properties of SHV-1 and Asn276Asp for Ampicillin, Piperacillin, Nitrocefin, and Cephalothin

	SHV-1	Asn276Asp
Ampicillin		
$K_m(\mu\text{M})$	183 ± 34	134 ± 13
$k_{\text{cat}}(\text{s}^{-1})$	3100 ± 300	989 ± 99
$k_{\text{cat}}/K_m(\mu\text{M}^{-1}\text{s}^{-1})$	17 ± 4	7 ± 1
Piperacillin		
$K_m(\mu\text{M})$	77 ± 7	105 ± 18
$k_{\text{cat}}(\text{s}^{-1})$	898 ± 90	976 ± 98
$k_{\text{cat}}/K_m(\mu\text{M}^{-1}\text{s}^{-1})$	12 ± 1	9 ± 1
Nitrocefin		
$K_m(\mu\text{M})$	21 ± 3	28 ± 3
$k_{\text{cat}}(\text{s}^{-1})$	237 ± 24	137 ± 14
$k_{\text{cat}}/K_M(\mu\text{M}^{-1}\text{s}^{-1})$	11 ± 2	5 ± 1
Cephalothin		
$K_m(\mu\text{M})$	29 ± 1	83 ± 11
$k_{\text{cat}}(\text{s}^{-1})$	14 ± 1	10 ± 1
$k_{\text{cat}}/K_m(\mu\text{M}^{-1}\text{s}^{-1})$	0.47 ± 0.03	0.12 ± 0.02

Table 3

Kinetic Properties of SHV-1 and Asn276Asp for Clavulanate, Cephalothin BATSI, Methylidene Penem, and Meropenem

	SHV-1	Asn276Asp
	Clavulanate	
$K_i(\mu\text{M})$	1.37 ± 0.23	7.44 ± 0.84
$K_I(\mu\text{M})$	0.72 ± 0.11	1.12 ± 0.11
$k_{\text{inact}} (\text{s}^{-1})$	0.015 ± 0.002	0.015 ± 0.002
$k_{\text{inact}}/K_I (\mu\text{M}^{-1} \text{s}^{-1})$	0.021 ± 0.004	0.013 ± 0.002
$k_{\text{cat}}/k_{\text{inact}} (t_n)$	40 ± 10	50 ± 10
$k_{\text{cat}} (\text{s}^{-1})$	0.60 ± 0.17	0.75 ± 0.18
	Achiral Cephalothin BATSI	
$K_i(\mu\text{M})$	42 ± 4	36 ± 4
	Chiral Cephalothin BATSI	
$K_i(\mu\text{M})$	0.68 ± 0.07	3.8 ± 0.4
	Methylidene Penem	
$K_i(\mu\text{M})$	0.057 ± 0.006	0.102 ± 0.001
$K_I(\mu\text{M})$	0.026 ± 0.004	0.039 ± 0.002
$k_{\text{inact}} (\text{s}^{-1})$	0.17 ± 0.01	0.24 ± 0.02
$k_{\text{inact}}/K_I (\mu\text{M}^{-1} \text{s}^{-1})$	6.5 ± 1.0	6.2 ± 0.6
$k_{\text{cat}}/k_{\text{inact}} (t_n)$	2 ± 1	2 ± 1
$k_{\text{cat}} (\text{s}^{-1})$	0.34 ± 0.17	0.48 ± 0.24
	Meropenem	
$K_i(\mu\text{M})$	31 ± 3	1411 ± 14

Table 4

Differences in Substrate and Inhibitor Binding Energies for SHV-1 and Asn276Asp

	<i>G</i> (kcal/mol)
<i>β</i> -Lactam Substrates	
ampicillin	-0.18
piperacillin	+ 0.18
cephalothin	+ 0.62
Inhibitors	
clavulanate	+ 1.00
achiral cephalothin BATSI	-0.09
chiral cephalothin BATSI	+ 1.02
methylidene penem	+ 0.34
meropenem	+ 2.26