Infections with bacteria that contain hydrolytic \( \beta \)-lactamase enzymes are becoming a serious problem in the United States. Mutations at Met-69, an amino acid proximal to the active site Ser-70 in the TEM-1 and SHV-1 \( \beta \)-lactamases, have emerged as a puzzling cause of bacterial resistance to inhibitors of \( \beta \)-lactamases. Site-saturation mutagenesis of the 69 position in SHV \( \beta \)-lactamase was performed to determine how mutations of this non-catalytic residue play a role in increasing 50% inhibitory concentrations (IC\(_{50}\) concentrations) for clinically important \( \beta \)-lactamase enzyme inhibitors. Two distinct phenotypes are evident in the variant \( \beta \)-lactamases studied: significantly increased minimum inhibitory concentrations (IC\(_{50}\) concentrations) for clavulanic acid for the Met69Ile, Leu, and Val substitutions, and unanticipated increased minimum inhibitory concentrations and hydrolytic activity toward ceftazidime, an advanced generation cephalosporin antibiotic, for the Met69Lys, Tyr- and Phe-substituted enzymes. Molecular modeling studies emphasize the conserved structure of these substitutions despite great variation in substrate specificity. This study demonstrates the key role of Met-69 in defining substrate specificity of SHV \( \beta \)-lactamases and alerts us to new phenotypes that may emerge clinically.

\( \beta \)-lactamases are serine hydrolases that act upon \( \beta \)-lactam antibiotics (Fig. 1), and thus prevent these drugs from interfering with cell wall synthesis in many types of bacteria. \( \beta \)-Lactamase production is the most important mechanism by which pathogenic Gram-negative bacteria become resistant to \( \beta \)-lactam antibiotics such as penicillins and cephalosporins (see Fig. 2). TEM-1 \( \beta \)-lactamase, first described in ampicillin-resistant Escherichia coli, is the most common plasmid-borne resistance enzyme in Class A (E.C.3.5.2.6). SHV \( \beta \)-lactamases are related to TEM in structure and function (64% homology) and are usually found to \( \beta \)-lactam-resistant Klebsiella pneumoniae. Some mutations have arisen in these enzymes under the selective pressure of antibiotic use, resulting in variants that are able to destroy or evade our most sophisticated drugs, including \( \beta \)-lactam-based “suicide inhibitors” like clavulanic acid (Fig. 2).

To date, 26 such inhibitor-resistant variants have been described including TEM and SHV enzymes with mutations at Ambler positions Arg-244, Met-69, Ser130, Asn-276 and Arg-275 (2, 3). In general, these residues are not directly involved in catalysis, although in the case of the Ser-130 the hydroxyl group is postulated to participate in cross-linking the mechanism based inhibitors to lead to irreversible inhibition (4–6).

The methionine at position 69 is not a highly conserved residue, although many Class A enzymes (3), including TEM-1 and SHV-1, contain this amino acid (7). Much is known about the Met-69 mutants of TEM \( \beta \)-lactamase (8, 9) including recent crystal structures of the M69I/M182T and M69V mutants (10), but less is known about such variants in SHV (11). From a crystal structure of wild type SHV-1 (12), it is known that the width of the substrate binding cavity is 0.7–1.2 Å larger than in TEM-1. The H10 helix residues (218–224) and the residues between the strands of \( \beta \)-pleated sheet such as Glu-240 have the largest deviations from the TEM structure. Also, SHV-1 naturally contains a Thr residue at position 182, which may act to stabilize the overall structure of the wild type and mutant proteins (10, 13). An additional E240K mutation in SHV may also act to stabilize the G238S cephalosporinase mutant, SHV-2.

In the case of TEM, several explanations for the observed inhibitor resistance have been put forth based on mutagenesis, kinetic, and molecular modeling experiments (9, 14–17). The two leading hypotheses are 1) that hydrolysis of the inhibitor is favored over irreversible inactivation (14) or 2) that decreased affinity for the inhibitor occurs as a result of displacement of a key region of the protein that borders the active site, the B3 strand of the \( \beta \)-pleated sheet (16, 17). Recently, new observations regarding indirect changes focused on Ser-130 have emerged as a possible explanation for the observed inhibitor resistance in the M69I and M69V mutants of TEM (10). Also, changes in the molecular dynamics of the M69L mutant of TEM have been modeled based on the recent crystal structure of this enzyme and may be at the heart of inhibitor resistance due to this substitution (18).

With the hypothesis that substitution with smaller, or similarly sized and more hydrophobic residues at position 69 might yield additional inhibitor-resistant enzymes for study based on possible movement of the B3 strand or indirect effects on other catalytically important residues, we set out to explore the role of this residue in defining substrate and inhibitor specificity in SHV. We used mutagenesis to create all the possible amino acid substitutions for Met-69 in SHV-1. These substitutions were then characterized in terms of phenotypic expression of antibiotic and inhibitor resistance in bacteria as well as levels of \( \beta \)-lactamase expression of the variant proteins. Selected enzymes were purified based on novel phenotypes and further characterized kinetically. An energy-minimized molecular modeling approach was undertaken to elucidate the structural changes giving rise to the
altered phenotypes. The unexpected finding of variants with selective cephalosporinase activity will be described.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—Bacterial strains and plasmids used have been described previously (11). A degenerate codon (NNS) at position Met-69 was used in the oligonucleotide primers constructed by Genosys Biotechnologies (The Woodlands, TX). The sequences of the primers were as follows: 5′-GAGAAACGCTTCAATTNNNSGCAGCGTTTAAGAAGTACG-3′ and 5′-CAGTCTTTTACGTGCTTNNNGATGAGGGAAAGCTTTATCATC-3′ where S can only be G or C.

Antibiotic Susceptibility—Antibiotics were obtained from the following companies: ampicillin, piperacillin, cephalothin (Sigma); nitrocefin (Becton-Dickinson, Cockeysville, MD); cefazidime (Glaxo-Wellcome); sodium tazobactam (Wyeth Pharmaceuticals, Pearl River, NY); lithium clavulanate (SmithKline Beecham, King of Prussia, PA); sulbactam (Pfizer, New York); ceftriaxone (Roche Molecular Biochemicals); and aztreonam (Bristol-Myers Squibb, Princeton, NJ). Minimum inhibitory concentrations for each of the Met-69 variant SHVs expressed in E. coli DH10B cells were determined using an LB agar dilution method as detailed previously (11). Reported MICs represent the median of three to five determinations and did not vary by more than a single dilution between determinations.

β-Lactamase Purification—β-Lactamases were liberated by stringent periplasmic fractionation (20). The crude lysate was purified in a two-step fashion as follows. First, preparative isoelectric focusing was done in Ultrodex granulated gel (Amersham Biosciences) using ampholines in the pH range of 3.5–10. Nitrocefin was used to locate the protein band in the gel. The protein was eluted with either 20 mM diethanolamine buffer, pH 8.3, or phosphate buffered saline (PBS), pH 7.4. The eluted protein solution was concentrated and further purified using a phenylboronate affinity column (MoBiTec, Marco Island, FL) as described previously (20). The β-lactamase was eluted with 10–40 ml of the 0.5 M borate (boric acid and NaOH), 0.5 M NaCl buffer, pH 7.0 (wild type, M69I, M69L; pH 8.3 (M69G, M69V); pH 10.0 (M69F)). Protein concentrations were determined using BioRad Protein Assay (BioRad, Hercules, CA), and purity was determined using an SDS-PAGE gel and was greater than 95%.

Kinetics—Kinetic constants $K_{cat}$ and $v_{cat}$ were determined by direct measurement of hydrolysis of substrates at room temperature in PBS, pH 7.4, using an Agilent™ 8402 diode array spectrophotometer. Measurements were made using wavelengths and extinction coefficients for nitrocefin, cefazidime, cephalothin, and ampicillin as previously described (11). Plots of velocity versus substrate concentration were fitted with a double exponential curve, and $IC_{50}$ concentrations are reported as ln (1/2)/exponent. The mean and standard deviation of three determinations were reported. Errors in calculated quantities such as catalytic efficiency and $\Delta \Delta G^\ddagger$ were estimated by calculating the variance (22).

Molecular Modeling—Energy-minimized structures of the M69F variant were created with Insight II software (Accelrys, Inc., San Diego, CA) using the Biopropyl and Discover 3 modules. The crystal structure of the SHV-1 β-lactamase (1SHV, Protein Data Bank, Rutgers, NJ) (12) was used to generate the M69F variant. The two lowest energy conformers of the variant as well as the original wild type structure were subjected to energy minimization to examine the effect of the minimization process. Structures were minimized including hydrogens and crystallographic waters using a pH of 7.4. The catalytically important Lys-73 was protonated, and the residue significant in the deacylation and possibly acylation of the enzyme (23), Glu-166, was deprotonated in the model structure. An active site zone was defined encompassing a radius of 15 Å, centered on the catalytic serine at position 70. Both the constant valence force field (CVFF) and AMBER force field were utilized. Van der Waals interactions were calculated using the cell-multipole method in the CVFF potential. In the AMBER potential, an atom-based method was used with a large 15-Å interaction radius. Coulomb interactions were calculated assuming a distance-dependent dielectric constant multiplied by a factor of 4 in both potentials. The region of the protein not including the active site was fixed, and the backbone atoms of the active site region were restrained by tethering to their starting coordinates using an anharmonic potential. The tethering force constant was varied to examine the effect of this restraint on the total energy of the system. Minimization was done stepwise, in 100-step increments, first calculating the steepest descents and then by conjugate gradient method (Polak-Ribiere) to find the final minimum given the large size of the system. The coordinates determined at convergence were used in the final energy-minimized structures. The structures of the wild type enzyme and the two lowest energy rotamer structures of the Phe variant were compared on the basis of their overall energies and the root mean square deviations (r.m.s.d.) of the active site backbone and side chain positions and evaluated using PROCHECK (24).

To compare the structures of the wild type and M69F enzymes with respect to ceftazidime binding, models were created using the ceftazidime structure from the Amp C enzyme (1IEL, Protein Data Bank) (25), and the crystal structure of SHV-1 inhibited with tazobactam (1G56, Protein Data Bank) (26). Four atoms of the opened β-lactam ring from the ceftazidime structure (C-7, C-8, N-5, and O-9) were initially superimposed with the corresponding atoms of the opened β-lactam ring of tazobactam (C-6, C-7, N-4, and O-8) to facilitate positioning of the molecule into the active site. Biopolymer was again used to generate the two lowest energy rotamers of the M69F substitution and to set the pH at 7.4. Crystallographic waters were used in the energy minimization that was undertaken as described above except that only the CVFF force field was utilized. This was necessary because several atom types

\[ \Delta \Delta G^\ddagger = \Delta G^\ddagger (SHV-1) - \Delta G^\ddagger (\text{mutant}) \]

\[ = E^\text{cat} \ln \left( \frac{K_{cat}}{K_{cat}(\text{mutant})} \right) \]  

(Fig. 2. Compounds used as B-lactamase substrates and inhibitors.)
found in the ceftazidime molecule could not be defined in the AMBER potential. Minimized structures of the wild type and Phe variant, with and without ceftazidime present, were compared.

RESULTS

**Mutagenesis**—Site-saturation mutagenesis yielded 18 of 19 substitutions at Ambler position 69. Site-directed mutagenesis was then used to successfully create the M69Q variant that was not found during screening.

**Minimum Inhibitory Concentrations**—Two distinct β-lactamase phenotypes (inhibitor-resistant and cephalosporinase enzymes) were found during the MIC testing. MICs for specific antibiotics are shown in Table I for the inhibitor-resistant variants (M69I,-L,-V), and in Table III for the cephalosporinase variants (M69F,-K,-Y). Overall, of the nineteen variants tested, none of the resistant variants (M69I, -L, -V, -F, -K, -Y) was found in the ceftazidime molecule could not be defined in the AMBER potential. Minimized structures of the wild type and Phe variant, with and without ceftazidime present, were compared.

**β-Lactamase Expression**—The level of expression was quite variable for each of the substituted enzymes, both in terms of the individual variation from experiment to experiment, and when comparing the relative amounts produced by each variant-containing strain (see Supplementary Data Table II at http://www.jbc.org). It is most important to note however that none of the resistant variants (M69I, -L, -V, -F, -K, -Y) was produced in excess of the wild type β-lactamase. The M69P enzyme was not produced in any measurable quantity.

**Kinetic Constants**—The Km and kcat values for the purified variant enzymes and wild type enzymes with their substrates are summarized in Tables II and IV, together with the calculated catalytic efficiencies and ΔΔG° values relative to wild type enzyme. With ampicillin, the majority of the variant enzymes studied retain their catalytic efficiency with the notable exception of the M69F protein. The M69I variant has a lower kcat than the wild type enzyme, but the catalytic efficiency is compensated by the reduction in Km. For ceftazidime, the only wild type and M69F enzymes have measurable rates of hydrolysis. For this substrate, the Phe variant demonstrates greater than 10-fold increased efficiency (relative kcat/Km = 10.4), driven by both increases in kcat and decreased Km.

**Molecular Modeling**—Fig. 3 depicts the overlay of the wild type and M69F variant and was generated by superimposing the backbone atoms of the original wild type crystal structure.

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**Table I**

| Met-69 substitution | Ampicillin | Ampicillin/clavulanate | Ampicillin/subactam | Ampicillin/tazobactam | Ceftazidime |
|---------------------|------------|------------------------|---------------------|-----------------------|-------------|
| Wild type, Met      | 16000      | 50/2.0                 | 50/64               | 50/32                 | 2.0         |
| Val                 | 8200       | 50/6.0                 | 50/130              | 50/64                 | 0.50        |
| Leu                 | 8200       | 50/4.0                 | 50/130              | 50/32                 | 0.50        |
| Ile                 | 8200       | 50/6.0                 | 50/130              | 50/64                 | 0.50        |

**Table II**

| Substrate | Protein       | Km (μM) | kcat (s⁻¹) | kcat/Km | ΔΔG° (kJ M⁻¹) |
|-----------|---------------|---------|------------|---------|---------------|
| Ampicillin| Ampicillin    | 150 ± 30| 2300 ± 300 | 15 ± 1  | 1             |
|           | M69I          | 39 ± 4  | 500 ± 100  | 10 ± 2  | 0.95          |
|           | M69L          | 110 ± 20| 1050 ± 40  | 10 ± 1  | 0.7           |
|           | M69V          | 80 ± 2  | 800 ± 100  | 10 ± 1  | 0.7           ||

**Table III**

| Met-69 substitution | Ampicillin | Ampicillin/clavulanate | Ceftazidime |
|---------------------|------------|------------------------|-------------|
| Wild type, Met      | 16,000     | 50/2.0                 | 2.0         |
| Lys                 | 510        | 50/0.03                | 4.0         |
| Phe                 | 510        | 50/0.06                | 8.0         |
| Tyr                 | 64         | 50/0                   | 4.0         |

**Table IV**

| Substrate | Protein       | Km (μM) | kcat (s⁻¹) | kcat/Km | ΔΔG° (kJ M⁻¹) |
|-----------|---------------|---------|------------|---------|---------------|
| Ceftazidime| Ampicillin   | 15 ± 4  | 0.21 ± 0.02| 0.014 ± .002| 1             |
|           | M69F         | 4.8 ± 0.1| 0.7 ± 0.1  | 0.15 ± 0.02| 10            |

μg/ml (data not shown). The bacteria containing the Pro substituted enzyme are highly susceptible to all antibiotics studied.
and the energy-minimized M69F structure. There is significantly less than 0.5 Å r.m.s.d. in their respective coordinates overall. On a per residue basis, no significant (>0.5 Å) deviations were noted for the side chain or backbone atoms when the minimized structures were compared. Despite the small structural changes (especially when compared with the overall resolution of the crystal structures on which the models are based), final energies of the wild type and M69F variant were not equivalent. The M69F enzyme was ~60 kcal/mol higher in energy using a CVFF potential. The AMBER minimizations gave a qualitatively similar result. Reduction in the tethering force constant used to restrain only the active site backbone atoms did not result in significant changes in the overall energy (<2 kcal/mol). The most significant differences, accounting for ~70% of the total energy difference, occurred in the non-bonded (repulsive and dispersive van der Waals) interactions, without any specific residues implicated. PROCHECK analyses of these structures showed that the stereochemistry of the wild type and Phe models was the same, with good quality Ramachandran plot statistics (93.5% of residues in most favored regions). Only two residues were in generously allowed regions: Tyr-105 and Met-(Phe) 69, irrespective of the model or the minimization procedure. No distorted geometries were noted for residues critical to catalytic activity, prior to or after the minimization procedures. The model depicting ceftazidime bound in the active site of the Phe variant is shown in Fig. 4 and shows the carboxylate group on the oxyimino side chain pointing away from the negatively charged Asp-104 and Glu-240 side chains at the opening of the active site cleft.

DISCUSSION

Methionine 69 is important in defining part of the "oxyanion hole" structure or attractive binding pocket in the SHV active site (17). Its side chain is buried in the hydrophobic core of the enzyme, and its dihedral angles are outside of energetically favorable conformations by Ramachandran plots (14). Many hypotheses (10, 14, 16, 17) exist regarding the exact role this residue plays in defining bacterial resistance to β-lactam antibiotics in strains containing these altered enzymes. Our study of SHV variants shows that two distinct enzyme phenotypes can result from amino acid substitutions at this position. The size of the residue at position 69 dramatically impacts on this substrate specificity. This comes at a high energetic cost to the Phe variant as demonstrated by our modeling studies.

Similar to TEM β-lactamase in SHV, only the Ile, Leu, and Val substitutions lead to inhibitor resistance, this despite the differences that are already present in the active site cavity size of SHV compared with TEM (12). Recent crystal structures of the TEM-32(M69I/M182T) and TEM-34(M69V) mutants at 1.61 and 1.52 Å resolution, respectively, show that the Ser-130 residue adopts a conformation in which it does not hydrogen bond as well with the Ser-70 in the apoenzyme (10). It also forms an additional hydrogen bond with Lys-73 (TEM-32). Whether a direct effect on the position of the Ser-70 may also occur, resulting in disruption of the positioning of an inhibitor...
intermediate with respect to Ser-130, is not clear. The structural
data reported shows differences on the order of 0.1–2.4 Å (greatest for the Ser-700y-Ser-1300y distance in the TEM-32 mutant compared with the M182T “wild type” mutant). Previous modeling studies of TEM have been unable to demonstrate these structural changes (9, 14). In contrast, a recent crystal structure of TEM M69L shows an overall r.m.s.d. of only 0.57 Å for the main chain atoms, and kinetic constants reveal the same preservation of function for this enzyme that we have observed for SHV (18). The explanation given for inhibitor resistance behavior in TEM M69L is that relative dynamic motion of Leu-69 and other active site residues is better correlated with movement of the Ω loop (Glu-166, Asn-170) than in the wild type structure. Unfavorable electrostatic interactions also exist that may prevent the usual positioning of the inhibitor C3 carboxylate with the Arg-244.

Such indirect effects of Met-69 mutations on the Ser-130 do not speak to affinity differences that might occur when β-lactams initially bind in the active site since Ser-130 is thought to interact mainly with the acyl enzyme intermediates (the Schiff base imine) (4–6). Our data indicate that the affinity of the variant SHV enzymes (both the inhibitor-resistant and cephalosporinase proteins) improves for a variety of β-lactam compounds, but affinity for clavulanic acid decreases for the inhibitor-resistant group. Ser-130 is also known to be involved in the slow, irreversible inhibition of the enzyme presumably by forming a cross-linked structure with a fragment of the inhibitor and the Ser-70 (4, 5, 26). However, given the small partition coefficient along this pathway (1, 27) and the timeframe of the bacterial lifespan, i.e. 20 min, it is unlikely that β-lactamase inhibitors have their clinical efficacy primarily via this irreversible pathway; rather, the transiently inhibited species may play a more significant role. Since hydrogen bonding by Ser-130 is felt to stabilize the acylation transition state structure rather than the initial Michaelis complex, this is where conformational changes in Ser-130, indirectly mediated by particular Met-69 substitutions, might play a role in resistance to inhibition. Whether this could also affect substrate specificity is less clear.

The presence of two conformations of Ser-70 in the TEM crystal structures is also intriguing given that the backbone amide of Ser-70 forms part of the oxyanion hole. That would explain the altered kinetic constants that we observed both in the inhibitor-resistant variants and the cephalosporinase variants. Similarly, more coordinated motion of the active site residues with the critical residues of the Ω loop in these variants could explain improved cephalosporinase activity as well as inhibitor resistance behavior.

Our modeling studies of M69F have again demonstrated the limitations of this methodology when based on crystal structures of finite resolution. We did not find evidence in our models for movement of Asp-104, the critical Gly-238 and Glu-240 residues, or the residues that make up part of the Ω loop, including Glu-166, and Asn-170, to explain the increased cephalosporinase activity of the enzyme. This occurred despite considerable efforts to allow free active site motion during minimization. However, despite only small structural changes, the overall energy of the M69F variant is considerably increased. This increase is somewhat starting in its magnitude given that the protein was extremely stable. The energetic “cost” of the mutation is not ameliorated by binding of the substrate; that is, the increase in the energy of the system on binding substrate is not less for the Phe variant than for the wild type enzyme. ΔG‡ is may be lower in the Phe variant than in the wild type because of its relatively less stable, higher energy ground state apoenzyme structure, assuming the same transition state structure for both.

In conclusion, these observations provide evidence of a unifying explanation regarding the role of the residue at position 69: its greatest effect is in determining substrate specificity. This is likely because of its uniquely constrained, high energy position proximal to the critical catalytic serine 70 and because of indirect effects on both the Ser-70 and Ser-130. Recent crystal structures of Met-69 mutants of TEM bear out the indirect effects of this residue on both catalytically important Ser residues. We have shown that the entire range of resistance phenotypes is possible by merely varying one residue within the β-lactamase enzyme. However, resistance comes at a cost of altered enzyme specificity and increased overall energy, particularly in the case of cephalosporinase phenotypes that lose most of their ampicillinase function and are ready targets for inhibitors of these enzymes. Crystal structures of these mutants with inhibitors in their active sites and of the M69F variant would be extremely valuable in further elucidating the impact of the Met-69 residue on protein structure and function.

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Unexpected Advanced Generation Cephalosporinase Activity of the M69F Variant of SHV \(\beta\)-Lactamase

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