Effects on Substrate Profile by Mutational Substitutions at Positions 164 and 179 of the Class A TEM*β-Lactamase from Escherichia coli*

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We investigated the effects of mutations at positions 164 and 179 of the TEM*β-Lactamase on turnover of substrates. The direct consequence of some mutations at these sites is that clinically important expanded-spectrum β-lactams, such as third-generation cephalosporins, which are normally exceedingly poor substrates for class A β-lactamases, bind the active site of these mutant enzymes more favorably. We employed site-saturation mutagenesis at both positions 164 and 179 to identify mutant variants of the parental enzyme that conferred resistance to expanded-spectrum β-lactams by their enhanced ability to turn over these antibiotic substrates. Four of these mutant variants, Arg*164 → Asn, Arg*164 → Ser, Asp*179 → Asn, and Asp*179 → Gly, were purified and the details of their catalytic properties were examined in a series of biochemical and kinetic experiments. The effects on the kinetic parameters were such that either activity with the expanded-spectrum β-lactams remained unchanged or, in some cases, the activity was enhanced. The affinity of the enzyme for these poorer substrates (as defined by the dissociation constant, Kd) invariably increased. Computation of the microscopic rate constants (kcat and k0) for turnover of these poorer substrates indicated either that the rate-limiting step in turnover was the deacylation step (governed by k0) or that neither the acylation nor deacylation became the sole rate-limiting step. In a few instances, the rate constants for both the acylation (kcat) and deacylation (k0) of the extended-spectrum β-lactam were enhanced. These results were investigated further by molecular modeling experiments, using the crystal structure of the TEM*β-Lactamase. Our results indicated that severe steric interactions between the large 7β functionalities of the expanded-spectrum β-lactams and the Ω-loop secondary structural element near the active site were at the root of the low affinity by the enzyme for these substrates. These conclusions were consistent with the proposal that the aforementioned mutations would enlarge the active site, and hence improve affinity.

Among clinical isolates of enteric Gram-negative bacilli, such as Escherichia coli, the most prevalent plasmid-encoded β-lactamase is the prototypical class A, or group 2b, enzyme, TEM-1 (1, 2). This enzyme is produced constitutively and is active principally against penicillins, such as ampicillin, rather than expanded-spectrum cephalosporins, monobactams, or carbapenems (2). Novel β-lactamases that confer resistance to such expanded-spectrum cephalosporins and monobactams in clinical isolates have arisen by mutation from class A β-lactamases, such as TEM or SHV (2–4). The most common alteration in these mutant enzymes is replacement of Arg*164, which is conserved among all “wild-type” class A β-lactamases. Many of these also contain other mutations. However, a single replacement of Arg*164 in TEM-1 by serine to produce the TEM-12 enzyme is by itself sufficient to enhance enzymatic activity against ceftazidime and aztreonam and thereby to confer clinically significant resistance to these β-lactams (5, 6).

Class A β-lactamases contain the Ω-loop, a conserved structural feature which consists of residues 164–179 (7–10). This structure, which forms a portion of the active-site pocket of the enzyme (9, 10), also contains the fully conserved Glu166, which promotes a water molecule for the deacylation step of β-lactam hydrolysis (9–16). From the crystal structures of class A β-lactamases (7–10), it is apparent that the base of the Ω-loop is anchored by a salt bridge between the side chains of Arg*164 and Asp*179, which are highly conserved residues in the amino acid sequences of known class A β-lactamases (17). It has been hypothesized that the poor activity of the wild-type TEM enzyme against expanded-spectrum β-lactams such as ceftazidime (a cephalosporin) and aztreonam (a monobactam) is due to steric hindrance by the Ω-loop of the bulky 7β side chains of these substrates in the active site (3, 18–20). In fact, we have shown, by molecular modeling with dynamics simulations and energy minimization, that such unfavorable steric interactions between the bulky 7β side chain function of cefepime, a new expanded-spectrum cephalosporin, and residues Pro*167 and Asn*170 of the TEM β-lactamase indirectly affect the second step of the catalytic mechanism (deacylation) (20).

Mutational replacements of either Arg*164 or Asp*179 that would disrupt the salt bridge between them might be expected to distort the Ω-loop (as was shown by the crystallographic structure of the Asp*179 → Asn mutant of the class A β-lactamase from Staphylococcus aureus PC1 (21)). Such restructured conformation of the Ω-loop might account for enhanced activity of mutant enzymes against expanded-spectrum β-lactams (20, 22) by better accommodating the bulky side chains of these substrates in the active site. We have previously generated mutants of the TEM*β-Lactamase with Asp*179 replaced by each of the 19 other amino acids to study the impact of disruption of the salt bridge at residue 179 on β-lactam resistance.

Production of β-lactamases is the most common mechanism of high-level resistance to β-lactam antibiotics in bacteria.

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TEM β-Lactamase Mutants at Arg164 or Asp179

conferred by the enzyme (23). Many of the mutational replacements of Asp179 led to a reduction in MIC1 of ampicillin and/or a rise in MIC of ceftazidime, but no rise in MIC of aztreonam, cefotaxime, or cefepime (expanded-spectrum cephalosporins), or imipenem (a carbapenem). We have extended herein that study on mutations of Asp179 to examine the effects of mutations of Arg164 at the other end of the salt bridge. We examined minimum inhibitory concentrations (MICs) of β-lactams conferred by all of the 19 mutant β-lactamases with amino acid replacements at this site. Furthermore, we then purified and studied the enzymes mutated at positions 164 or 179 that have the greatest effects on resistance to expanded-spectrum β-lactams to assess the structural and kinetics consequences of those mutations that lead to the observed resistance.

EXPERIMENTAL PROCEDURES

β-Lactams—Benzylpenicillin, ampicillin, pipercillin, cephaloridine, cephalothin, and cephalosporin C were purchased from Sigma. [2-[(Dimethylamino)phenyl]azo]-pyridinocephalosporin (PADAC) was a product of Calbiochem. Other β-lactams were gifts from the following: ceftazidime, Gleece; cephalosporin and aztreonam, Bristol-Myers Squibb; cefotaxime, Hoechst-Marion-Roussel; ceftaxalone, Roche Laboratories; imipenem, Merck and Co.

Plasmids and Host Bacterial Strain—E. coli JM83 (ara Δlac-proAB) rpsL (ara Zama15) (24) was used as the host for all plasmids. Plasmid pUC19-κacβ was constructed by cloning the kanamycin resistance gene aphA1 from Tn903 (25) into the BamHI site in the polylinker of pUC19 (24). This plasmid encodes the parental enzyme for this study, TEM-1,β-lactamase, which differs from the TEM-1 enzyme (26) by two mutations, Val23 → Ile and Ala164 → Val, which are considered to be enzymologically inconsequential (27). (The numbering of amino acid residues in the β-lactamase sequence is according to the convention of Ambler et al. (1).) Nineteen mutant transformants of E. coli JM83, each with a different replacement of Asp179 in TEM-1,β-lactamase were reported by Vakulenko et al. (23).

Site-directed Mutagenesis of TEM-1,β-lactamase and Nucleotide Sequencing—Random site-directed mutagenesis of Arg164 in the parental TEM-1,β-lactamase was carried out for that of Asp179 (22), except that the mutagenic oligonucleotide mixture contained all four bases at each of the three positions corresponding to the codon for residue 164. After mutagenesis, E. coli JM83 was transformed with mutated DNA by electroporation, and transformants were selected by growth on Mueller-Hinton agar plates containing 30 μg of kanamycin/ml.

DNA for nucleotide sequencing was isolated by an alkaline method (28). The DNA sequence was obtained by the method of Sanger et al. (29) using a Sequenase version 2.0 DNA sequencing kit (U.S. Biochemicals, Cleveland, OH) and (α-35S)dATP (NEN Life Science Products, Boston, MA). Mutant β-lactamases were identified according to the sequence of the codon in the bla gene corresponding to residue 164 of the TEM-1,β-lactamase and the entire β-lactamase gene and its promoter region on each mutant plasmid were sequenced. Only mutants with a single replacement at that site and no mutations elsewhere in the bla gene or its promoter were utilized for the study of the β-lactamase gene and its promoter region on each mutant plasmid.

Susceptibility Testing—The MICs of β-lactams for E. coli JM83 and its various transformants were determined by the microbroth dilution method in Mueller-Hinton broth with inocula of 105 colony forming units/ml and an 18-h incubation at 37 °C. Each MIC is the result of at least five determinations.

β-Lactamase Purification—β-Lactamases were purified to homogeneity (as judged by SDS-polyacrylamide gel electrophoresis) from E. coli JM83 transformants bearing the parental TEM-1,β-lactamase or mutant enzymes with a replacement of Arg164 or Asp179. One-liter cultures of each transformant grown overnight in Terrific Broth (Life Technologies, Inc.) served as the source of enzymes for purification. The procedure for enzyme purification was according to Zafaralla et al. (30).

Kinetics Measurements—Kinetics measurements were carried out by continuous spectrophotometric assays at room temperature in a Hewlett-Packard 452 diode-array instrument, as described by Imtiaz et al. (22) and Taibi-Tronche et al. (20). The values of Vmax and Km were determined from the Lineweaver-Burk plots of the initial steady-state velocities for each enzyme with the following ranges of substrate concentrations: ampicillin (50–200 μM), pipercillin (20–100 μM), cephalothin (80–200 μM), cephalosporin C (90–240 μM), cephaloridine (100–250 μM), aztreonam (200–500 μM), cefotaxime (30–200 μM), and ceftazidime (20–100 μM). Initial rates were determined from the first 5–10% of the reaction with five to seven substrate concentrations generally flanking the Km, values.

Determination of the Dissociation Constants and Calculation of the Microscopic Rate Constants—The procedure developed by Zafaralla et al. (30) was followed to determine the dissociation constant (Kd) for ceftazidime and ceftazidime with the TEM-1,β-lactamase and its Asn164 and Gly179 mutants. The values for Kd were also determined for ceftazidime with the Ser164 and Asn179 mutant enzymes. Hydrolysis of PADAC was monitored at 610 nm (Δε50 = 1. 240 M−1 cm−1). Two concentrations of PADAC (200 and 300 μM) were used. A series of mixtures containing both PADAC and at least five concentrations of various substrates flanking the dissociation constant were prepared in 100 mM sodium phosphate buffer, pH 7.0, in a total volume of 0.3 ml. A portion of enzyme was added to start the inhibition assays. Enzyme inhibition by the given substrate, in each case was invariably competitive in nature. The values for the acylation rate constants (kac) and deacylation rate constants (kd) for ceftazidime and cephalaxin with TEM-1,β-lactamase and its mutants were measured by following the method described by Taibi-Tronche et al. (20).

Enzyme Activity after Hydrolysis of Ceftazidime and Cefotaxime—Aromatic β-lactamase and its mutant variants were used for energy minimization and structure determination. The crystal coordinates were used as starting conformation for the proteins. Small molecules were energy-minimized using the MOPAC program (AM1 parameterization). MOPAC MNDO electrostatic potential charges were used as a reference to develop charges on the proteins. Small molecules were energy-minimized using the AMBER software package was used for energy minimization and structure determination. The procedure developed by Zafaralla et al. (30) was followed to determine the dissociation constant (Kd) for ceftazidime and ceftazidime with the TEM-1,β-lactamase and its Asn164 and Gly179 mutants. The values for Kd were also determined for ceftazidime with the Ser164 and Asn179 mutant enzymes. Hydrolysis of PADAC was monitored at 610 nm (Δε50 = 1. 240 M−1 cm−1). Two concentrations of PADAC (200 and 300 μM) were used. A series of mixtures containing both PADAC and at least five concentrations of various substrates flanking the dissociation constant were prepared in 100 mM sodium phosphate buffer, pH 7.0, in a total volume of 0.3 ml. A portion of enzyme was added to start the inhibition assays. Enzyme inhibition by the given substrate, in each case was invariably competitive in nature. The values for the acylation rate constants (kac) and deacylation rate constants (kd) for ceftazidime and cephalaxin with TEM-1,β-lactamase and its mutants were measured by following the method described by Taibi-Tronche et al. (20).

Stability of β-Lactamases—A mixture containing 330 nm β-lactamase (TEM-1,β-lactamase or various mutant enzymes) and 240 nm theromolin was incubated at 46 °C. At regular time intervals, 5-μl aliquots were added to 995 μl of 2 mM benzylpenicillin in 100 mM sodium phosphate buffer, pH 7.0. The level of residual activity of the enzyme was then measured for the time of incubation with theromolin. A control experiment in which no theromolin was added to the incubation mixture was carried out to measure any loss of activity due to heat alone.

Computational Models—The Tripos software Sybyl was used for structure visualization and molecular modeling. The AMBER software package was used for energy minimization and structure determination. The crystal coordinates were used as starting conformations for the proteins. Small molecules were energy-minimized using the MOPAC program (AM1 parameterization). MOPAC MNDO electrostatic potential charges were used as a reference to develop charges on non-peptide portions of the complexes. The AMBER force field parameters were used for energy minimization, and missing force field parameters were developed for non-peptide substrate portions of the complex. The entire complex was energy-minimized until one of the following conditions was reached: 1) total number of the iterations reached 10,000; 2) the energy difference for two successive cycles was less than 0.05 kcal/mol; or 3) the gradient norm for two consecutive iterations was less than 0.05 kcal/mole of A.

RESULTS

Susceptibilities to β-Lactams of Transformants Bearing TEM-1,β-lactamase and Each Mutant Derivative with a Replacement of Arg164—Table I presents the susceptibilities of the background host strain, E. coli JM83, alone or bearing plasmids that encode the parental TEM-1,β-lactamase or each of the 19 mutant enzymes resulting from replacement of Arg164. We have listed the mutant enzymes in decreasing order of ampicillin MICs that they confer. Although none of the mutant enzymes confers resistance to ampicillin quite as high as that produced by the parental enzyme, most of them produce a high level of ceftazidime resistance. Only five of the mutant enzymes confer MICs of ampicillin less than 1,000 μg/ml (the MICs of the wild-type TEM-1,β-lactamase and its Asn164, Asp179 MICs of 32,000 μg/ml conferred by the parental enzyme).

Although the parental enzyme has little effect on the susceptibility to ceftazidime, all but four of the mutants show at least a 4-fold rise in MIC over that (0.5 μg/ml) of the strain containing the parental enzyme. Seven of the mutants confer ceftazidime MICs ≥ 4 μg/ml, and the three mutants (Ser164, Asn164,
TEM β-Lactamase Mutants at Arg<sup>164</sup> or Asp<sup>179</sup>

**Table I**

Susceptibilities to β-lactams of E. coli JM83 alone and producing parental TEM<sub>pUC19</sub> β-lactamase or mutant enzymes with replacements of Arg<sup>164</sup>

| Amino acid | AMP µg/ml | CAZ µg/ml | CEP µg/ml | AZT µg/ml | TAX µg/ml | TRX µg/ml |
|------------|------------|------------|------------|------------|------------|------------|
| None<sup>b</sup> | 4 | 0.12 | 0.015 | 0.06 | 0.03 | 0.03 |
| Arg | 32,000 | 0.25 | 0.12 | 0.12 | 0.03 | 0.06 |
| Ser | 16,000 | 16 | 2 | 1 | 0.25 | 0.25 |
| Asn | 8,000 | 32 | 4 | 4 | 0.5 | 0.5 |
| His | 8,000 | 8 | 0.5 | 0.5 | 0.12 | 0.12 |
| Asp | 4,000 | 4 | 0.25 | 0.12 | 0.06 | 0.06 |
| Gly | 4,000 | 4 | 0.25 | 0.12 | 0.06 | 0.06 |
| Gln | 4,000 | 4 | 0.12 | 0.06 | 0.03 | 0.06 |
| Thr | 4,000 | 1 | 0.06 | 0.06 | 0.03 | 0.06 |
| Ala | 2,000 | 4 | 0.12 | 0.12 | 0.06 | 0.06 |
| Cys | 2,000 | 2 | 0.12 | 0.06 | 0.03 | 0.03 |
| Glu | 2,000 | 2 | 0.06 | 0.06 | 0.06 | 0.06 |
| Lys | 2,000 | 1 | 0.03 | 0.03 | 0.03 | 0.06 |
| Met | 2,000 | 1 | 0.06 | 0.06 | 0.03 | 0.06 |
| Leu | 1,000 | 2 | 0.03 | 0.03 | 0.03 | 0.06 |
| Phe | 1,000 | 0.5 | 0.03 | 0.03 | 0.03 | 0.06 |
| Tyr | 250 | 1 | 0.03 | 0.03 | 0.03 | 0.06 |
| Trp | 125 | 0.5 | 0.03 | 0.06 | 0.03 | 0.06 |
| Val | 125 | 0.5 | 0.03 | 0.06 | 0.03 | 0.06 |
| Ile | 32 | 0.5 | 0.015 | 0.06 | 0.03 | 0.06 |
| Pro | 8 | 1 | 0.03 | 0.06 | 0.03 | 0.06 |

<sup>a</sup> AMP, ampicillin; CAZ, ceftazidime; CEP, cefepime; AZT, aztreonam; TAX, cefotaxime; TRX, ceftriaxone.

<sup>b</sup> E. coli JM83.

and His<sup>164</sup>) which produce the highest residual MICs of ampicillin also confer the highest MICs of ceftazidime (16, 32, and 8 µg/ml, respectively).

Although little effect on cefepime susceptibility is produced by most of the mutant enzymes, the three that produce the highest levels of resistance to ceftazidime also confer MICs of cepafune at 4-fold higher than that of the strain bearing the parental enzyme. The Ser<sup>164</sup> and Asn<sup>164</sup> mutants have cepafune MICs 16- and 32-fold greater than that of the strain with the parental enzyme, but the resulting MICs are still only 2 and 4 µg/ml, respectively. These same mutants also have elevations of MICs for aztreonam, although to only 1 µg/ml. Likewise, only the Ser<sup>164</sup> and Asn<sup>164</sup> mutants produce at least a 4-fold rise in MIC of both cepafune and ceftriaxone over that of the strain with the parental enzyme, but the resulting MICs of both cephalosporins are only 0.25 and 0.5 µg/ml, respectively, with the two mutant strains. Neither the parental TEM<sub>pUC19</sub> enzyme nor any of the mutant derivatives with a replacement of Arg<sup>164</sup> or Asp<sup>179</sup> conferred an elevated MIC of imipenem (data not shown).

**Kinetics Parameters of the TEM<sub>pUC19</sub> β-Lactamase and Its Mutants That Have Replacement of Arg<sup>164</sup> by Asn (Arg<sup>164</sup> → Asn) or Ser (Arg<sup>164</sup> → Ser) or Bear Replacement of Asp<sup>179</sup> by Asn (Asp<sup>179</sup> → Asn) or Gly (Asp<sup>179</sup> → Gly)—**The steady-state kinetics parameters with the TEM<sub>pUC19</sub> β-lactamase and several of its mutant variants (Table II) were determined for three penicillins (penicillin G, ampicillin, and piperacillin), for three narrow-spectrum cephalosporins (cephaloridine, cephalothin, and cephalosporin C), for a monobactam (aztreonam), and for two expanded-spectrum cephalosporins (ceftaxime and ceftriaxime), as a complement to the comparable experiments with cepafune that were reported earlier (20). The penicillins are turned over by the parental TEM<sub>pUC19</sub> β-lactamase with k<sub>cat</sub>/K<sub>m</sub> in the order of 10<sup>7</sup> M<sup>-1</sup> s<sup>-1</sup>, and the corresponding catalytic efficiency for the narrow-spectrum cephalosporins is 1–3 orders of magnitude lower. Aztreonam, cefotaxime, ceftazidime, and cefepime are turned over by the TEM<sub>pUC19</sub> β-lactamase with still much lower k<sub>cat</sub>/K<sub>m</sub> values (7.4 × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1</sup>, 7.8 × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1</sup>, 40 M<sup>-1</sup> s<sup>-1</sup>, and 2.5 × 10<sup>5</sup> M<sup>-1</sup> s<sup>-1</sup>, respectively). Among these four expanded-spectrum β-lactams, ceftazidime exhibits the smallest turnover number (k<sub>cat</sub> = 0.008 ± 0.003 s<sup>-1</sup>), which is 22- and 50-fold lower than the turnover numbers for ceftriaxime and aztreonam, respectively. These values contrast with those for penicillins, which are in the range of 770–1570 s<sup>-1</sup> and those for the narrow-spectrum cephalosporins, which are in the range of 33–780 s<sup>-1</sup>. The slow turnover of these poor substrates is believed to be due to the bulky group at the C<sub>7</sub>-position, which has unfavorable steric interactions with the Ω-loop that spans residues 164–179 of the TEM<sub>pUC19</sub> β-lactamase (see below).

Among the mutant β-lactamases with replacements of Arg<sup>164</sup>, those with an Arg<sup>164</sup> → Ser or Arg<sup>164</sup> → Asn mutation confer the highest levels of resistance to ceftazidime; furthermore, they are associated with the greatest increases in MIC against the other β-lactams tested, even though the mutant enzymes remain nominally susceptible to these other β-lactams by usual clinical criteria. Therefore, the kinetics of these mutant enzymes were studied in comparison with those of the parental TEM<sub>pUC19</sub> enzyme. Such replacements of Arg<sup>164</sup> would be expected to disrupt the salt bridge between Arg<sup>164</sup> and Asp<sup>179</sup> that anchors the base of the Ω-loop. Since we had previously examined the effects of mutational replacement of Asp<sup>179</sup> in the TEM<sub>pUC19</sub> enzyme on susceptibility to various β-lactams (23), we included in these kinetics studies the two mutants with replacements of Asp<sup>179</sup>, Asp<sup>179</sup> → Asn and Asp<sup>179</sup> → Gly, which produced the greatest increases in MIC of ceftazidime.

As seen in Table II, the Arg<sup>164</sup> → Asn mutation has a modest effect (less than 5-fold reduction) on the efficiency of hydrolysis (k<sub>cat</sub>/K<sub>m</sub>) of the penicillin and narrow-spectrum cephalosporin substrates, in comparison with the TEM<sub>pUC19</sub> enzyme. The Arg<sup>164</sup> → Ser mutation has a somewhat greater effect (10–100-fold reduction from that for the parental enzyme) with these substrates than the mutation to Asn at that site. In contrast to the impairment of catalytic efficiency with the penicillin and narrow-spectrum cephalosporin substrates produced by the Arg<sup>164</sup> → Asn mutation, that mutation raises the efficiency of hydrolysis of the expanded-spectrum β-lactams (increases over TEM<sub>pUC19</sub> of 16, 29, 675, and 364-fold for aztreonam, ceftaxime, ceftazidime, and cefepime, respectively). The Arg<sup>164</sup> →
Ser mutation also increases the catalytic efficiency, but to a lesser extent (increases over TEM<sub>pUC19</sub> of 3-, 1.4-, and 13-fold for aztreonam, cefotaxime, and cefadroxil, respectively). The two mutations replacing Asp<sup>179</sup> or Asp<sup>179</sup> to Gly, produce a greater decline in the efficiency of hydrolysis of the penicillin and narrow-spectrum cephalosporin substrates than the mutations replacing Arg<sup>164</sup>. In contrast to the effects of the mutations of Arg<sup>164</sup>, the mutations replacing Asp<sup>179</sup> did not raise the efficiency of hydrolysis of aztreonam, cefotaxime, or cefadroxil over that of the parental enzyme, but each produced a 10-fold increase in catalytic efficiency for cefadroxil.

The dissociation constant ($K_d$), which indicates the affinity of an enzyme for a substrate, was measured for several of the expanded-spectrum β-lactams with the TEM<sub>pUC19</sub> β-lactamase and its mutants that have replacements at positions 164 and 179 (Table III). The results with cefepime (20) are also included for comparison. The values of $K_d$ with the TEM<sub>pUC19</sub> β-lactamase are high with aztreonam and the three expanded-spectrum cephalosporins (range 3.7–10.3 mM), reflecting the low affinity of this enzyme for these substrates. The Arg<sup>164</sup>→Asn mutant has much lower $K_d$ values for both cefotaxime and cefepime; the $K_d$ for cefadroxil is reduced, but not nearly to the same extent. The Arg<sup>164</sup>→Ser mutant enzyme also has a comparable, moderately reduced $K_d$ for cefadroxil. The Asp<sup>179</sup>→Gly mutant has a marked reduction of $K_d$ for cefadroxil as well as for the other expanded-spectrum cephalosporins and

### Table II

| Substrate | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|-----------|----------|------|--------------|
| Penicillin G | TEM<sub>pUC19</sub> | 1570 ± 300 | 120 ± 30 |
|            | R164N    | 153 ± 3  | 37 ± 3  |
|            | R164S    | 21.9 ± 0.4 | 24 ± 1.4 |
|            | D179N    | 1.52 ± 0.4 | 16 ± 3  |
|            | D179G    | 0.72 ± 0.02 | 39 ± 2  |
| Ampicillin  | TEM<sub>pUC19</sub> | 1040 ± 120 | 27 ± 5   |
|            | R164N    | 79 ± 1   | 7.1 ± 0.7 |
|            | R164S    | 15 ± 1   | 37 ± 4   |
|            | D179N    | 1.60 ± 0.03 | 85 ± 3  |
|            | D179G    | 1.4 ± 4  | 1100 ± 400 |
| Piperacillin| TEM<sub>pUC19</sub> | 770 ± 10 | 70 ± 10  |
|            | R164N    | 109 ± 2  | 47 ± 3   |
|            | R164S    | 11 ± 1   | 22 ± 2   |
|            | D179N    | 0.74 ± 0.04 | 55 ± 7  |
|            | D179G    | 0.43 ± 0.01 | 31 ± 2  |
| Cephloridine | TEM<sub>pUC19</sub> | 780 ± 240 | 670 ± 200 |
|            | R164N    | 65 ± 1   | 89 ± 3   |
|            | R164S    | 22 ± 2   | 239 ± 25 |
|            | D179N    | 1.3 ± 0.2 | 900 ± 100 |
|            | D179G    | 6 ± 0.6  | 870 ± 100 |
| Cephalosporin C | TEM<sub>pUC19</sub> | 33 ± 3   | 950 ± 80  |
|            | R164N    | 10 ± 4   | 850 ± 200 |
|            | R164S    | 1.2 ± 0.02 | 440 ± 90 |
|            | D179N    | 0.062 ± 0.002 | 38 ± 7  |
|            | D179G    | 0.69 ± 0.04 | 120 ± 13 |
| Cephalothin  | TEM<sub>pUC19</sub> | 41 ± 5   | 210 ± 30  |
|            | R164N    | 41 ± 5   | 455 ± 53  |
|            | R164S    | 3.5 ± 0.2 | 180 ± 15  |
|            | D179N    | 0.21 ± 0.01 | 48 ± 2   |
|            | D179G    | 0.31 ± 0.01 | 41 ± 4   |
| Aztreonam   | TEM<sub>pUC19</sub> | 0.4 ± 0.1 | 550 ± 200 |
|            | R164N    | 6.7 ± 0.2 | 540 ± 15  |
|            | R164S    | 1.5 ± 0.2 | 635 ± 65  |
|            | D179N    | 0.06 ± 0.01 | 220 ± 30 |
|            | D179G    | 0.06 ± 0.01 | 340 ± 70 |
| Cefotaxime  | TEM<sub>pUC19</sub> | 0.18 ± 0.04 | 230 ± 50  |
|            | R164N    | 5.9 ± 0.7 | 257 ± 36  |
|            | R164S    | 0.20 ± 0.02 | 174 ± 15  |
|            | D179N    | 0.023 ± 0.001 | 29 ± 3  |
|            | D179G    | 0.007 ± 0.001 | 102 ± 16 |
| Ceftazidime | TEM<sub>pUC19</sub> | 0.008 ± 0.003 | 200 ± 100 |
|            | R164N    | 8 ± 2    | 296 ± 60  |
|            | R164S    | 1.4 ± 0.2 | 270 ± 80  |
|            | D179N    | 0.042 ± 0.008 | 110 ± 20 |
|            | D179G    | 0.041 ± 0.003 | 96 ± 9   |
| Cefepime<sup>a</sup> | TEM<sub>pUC19</sub> | 0.6 ± 0.1 | 240 ± 50  |
|            | R164N    | 290 ± 30 | 317 ± 28  |
|            | R164S    | 22 ± 2   | 230 ± 17  |
|            | D179G    | 0.024 ± 0.001 | 15 ± 2   |

<sup>a</sup> Previously reported in Ref. 20.
aztreonam. The other mutant with replacement of Asp\textsuperscript{179}, Asp\textsuperscript{179} → Asn, also had a marked reduction in $K_a$ for ceftazidime.

We were able to calculate the microscopic rate constants for acylation and deacylation for hydrolysis of aztreonam and the expanded-spectrum cephalosporins by the TEM\textsubscript{pUC19} β-lactamase and the mutant derivatives that were studied kinetically (Table III), from the dissociation constant and the steady-state parameters. For the TEM\textsubscript{pUC19} β-lactamase, the $k_2$ for each of these substrates is greater than $k_3$, and $k_3$ in turn is similar to the corresponding $k_{cat}$ value. Thus, deacylation appears to be the rate-limiting step for this enzyme. As with the TEM\textsubscript{pUC19} β-lactamase, for all of the interactions of the mutant enzymes with the various substrates, $k_3$ is similar to the corresponding $k_{cat}$. For those mutant enzymes where the values for $k_2$ and $k_3$ approach each other, however, one could not assign a unique rate-limiting step. In others, the rate-limiting step was deacylation. However, for none of the mutant enzymes was acylation rate-limiting step. In others, the rate-limiting step was deacylation for hydrolysis of aztreonam and the cephalosporins, such as ceftazidime, undergoing slow acylation and deacylation in the active site of the enzyme (Table III), by the general pathway shown in Scheme 1.

Cephalosporins, such as ceftazidime, were especially designed to resist the action of β-lactamases by what turns out to be the unfavorable steric interactions of their bulky 7β side chain functions. The acyl-enzyme intermediates were modeled for ceftazidime in the active site of the TEM\textsubscript{pUC19} β-lactamase in order to explain the structural bases for its slow rate of hydrolysis by the TEM\textsubscript{pUC19} β-lactamase. We have determined that the rate of deacylation is sufficiently slow that the $R_2$ group at the C₃ position departs from ceftazidime before deacylation of the enzyme takes place (Scheme 1). Therefore, the acyl-enzyme species C (Scheme 1) was modeled for ceftazidime. In modeling of the structure(s) for the acyl-enzyme intermediate(s), two starting conformations were used with different orientations of the 7β side chain. Both positions were similar to the two crystallographic structures for the acyl-enzyme intermediates of the DD-peptidase/transpeptidase (a penicillin-binding protein) with cefotaxime and cephahlothin, third- and first-generation cephalosporins, respectively (structures 1cef and 1ceg from the Brookhaven Protein Data Bank, Ref. 31). The DD-peptidase/transpeptidase has some sequence and domain similarity to the DD-peptidase/transpeptidase (a penicillin-binding protein) with cefotaxime and cephahlothin, and also that this functionality would cause the deacylation step to be rate-limiting (Table III). This finding was supported by modeling experiments, where steric interactions of the 7β group were highly unfavorable. However, it is significant to note that the two independent energy-minimization procedures converged to only one conformation, indicating a single conformational possibility for the acyl-enzyme species C. The energy-minimized structure is shown in Fig. 1.

Our previous modeling for cefepime had shown that the bulky 7β side chain was sterically encumbered by residues.

| β-Lactamase | Substrate | $K_a$ | $k_2$ | $k_3$ |
|-------------|-----------|------|------|------|
| TEM\textsubscript{pUC19} | Aztreonam | 3.7 ± 0.1 | 2.7 ± 1 | 0.47 ± 0.21 |
| | Cefotaxime | 3.8 ± 0.3 | 3.0 ± 0.3 | 0.2 ± 0.05 |
| | Ceftazidime | 9.9 ± 1.0 | 0.39 ± 0.01 | 0.008 ± 0.003 |
| | Cefepime\textsuperscript{a} | 10.3 ± 0.8 | 26 ± 7 | 0.6 ± 0.2 |
| R164N | Cefotaxime | 0.56 ± 0.06 | 13 ± 2 | 11 ± 3 |
| | Ceftazidime | 3.3 ± 0.2 | 89 ± 9 | 9 ± 1.3 |
| | Cefepime\textsuperscript{a} | 0.7 ± 0.1 | 635 ± 117 | 535 ± 130 |
| R164S | Ceftazidime | 4.3 ± 0.4 | 22.4 ± 5.6 | 1.5 ± 0.5 |
| D179G | Aztreonam | 0.38 ± 0.04 | 0.07 ± 0.02 | 0.06 ± 0.01 |
| | Cefotaxime | 0.31 ± 0.02 | 0.02 ± 0.004 | 0.01 ± 0.002 |
| | Ceftazidime | 0.15 ± 0.01 | 0.12 ± 0.01 | 0.061 ± 0.007 |
| | Cefepime\textsuperscript{a} | 1.7 ± 0.2 | 2.7 ± 0.5 | 0.024 ± 0.006 |
| D179N | Ceftazidime | 0.20 ± 0.03 | 0.078 ± 0.08 | 0.074 ± 0.01 |

\textsuperscript{a}Previously reported in Ref. 20.
Asn\textsuperscript{170} and Pro\textsuperscript{167}, located within the \( \Omega \)-loop Fig. 2, resulting in displacement of the hydrolytic water by approximately 0.5 Å from its usual position. The model of ceftazidime depicted in Fig. 1 reveals that the 7\( \beta \) side chain experiences unfavorable steric interactions with the active site, which is even more significant in the case of ceftazidime than that of cefepime. We used the three available crystal structures for the TEM\textsubscript{puc19} \( \beta \)-lactamase for comparison to our model. These reference crystal structures are for the native TEM-1 enzyme (10) and the two active-site acylated derivatives, one modified by 6\( \alpha \)-hydroxymethylpenicillanate (15) and another by penicillin G in the case of the deacylation-deficient Glu\textsuperscript{166} \( \rightarrow \) Asn mutant (9). The superimposition of the coordinates for the C\( \alpha \) of the active sites of these three crystallized enzymes with our model revealed

**Scheme 1.** The general mechanism for hydrolysis of the expanded-spectrum cephalosporins by class A \( \beta \)-lactamases.

**Fig. 1.** Stereoview of the energy-minimized structure for the acyl-enzyme intermediate for ceftazidime in the active site of the TEM\textsubscript{puc19} \( \beta \)-lactamase. The substrate portion of the complex is shown in bold.

| Compound | \( R_1 \) | \( R_2 \) |
|----------|----------|----------|
| Cefepime | \text{[Structure Image]} | \text{[Structure Image]} |
| Cefotaxime | \text{[Structure Image]} | \text{[Structure Image]} |

\( R_1 \) and \( R_2 \) represent the chemical structures of the cephalosporins as shown in the respective images.
that the hydrolytic water molecule in our model is at the same location as in the penicillin G complex with the Glu$^{166} \rightarrow$ Asn mutant. It is displaced more than 1 Å from the positions for the hydrolytic water in the wild-type enzyme and from that for the enzyme acylated by 6a-hydroxymethylpenicillinate. Moreover, as shown in Scheme 2, the unfavorable interactions cause the hydrolytic water to compete with the amide proton of the Asn$^{170}$ for hydrogen bonding to the same oxygen of Glu$^{166}$. This competition weakens the ability of Glu$^{166}$ to serve as the general base promoting the hydrolytic water for deacylation. Therefore, in the absence of proper activation of the hydrolytic water, the enzyme is impaired for effective deacylation of the acyl-enzyme intermediate of ceftazidime. Indeed, these findings are supported by the results, showing the slow and rate-limiting deacylation seen for ceftazidime. Furthermore, the high value of $K_d$ for ceftazidime indicates the low affinity of this substrate for the class A $\beta$-lactams, which is due to the unfavorable steric interaction of the 7β side chain.

Fig. 3 shows the superimposition of the catalytic serine acylated by ceftazidime in the conformation predicted by our modeling experiments and the catalytic serine of the class C $\beta$-lactamase from Citrobacter freundii modified by aztreonam. The crystal structure of the latter was determined previously (32), but has not been made available to the public. We used the scanned stereo picture from the published work by Oefner et al. (32). Subsequently, we entered the coordinates of this structure in pixels into a computer. A program was written by us to convert these coordinates in pixels for two images (left and right) into a three-dimensional structure. Classes A and C of $\beta$-lactamases share a topological similarity that is even more significant for their active sites. Therefore, one can expect that ceftazidime and aztreonam would bind in a similar manner to the active sites of these enzymes, certainly so for their identical 7β side chains. Indeed, one can see that the structures for these side chains are essentially identically sequestered within the active sites of these enzymes (Fig. 3). This observation bolsters the reliability of the model for the acyl-enzyme intermediate for ceftazidime reported here.

**DISCUSSION**

Expanded-spectrum cephalosporins are mainstays in clinical treatment of bacterial infections. Frequent use of these drugs has led to selection of mutant variants of $\beta$-lactamases with broadened substrate spectra that include extended-spectrum $\beta$-lactamases (33). A number of the amino acid residues that undergo mutational substitutions in parental enzymes to produce these extended-spectrum $\beta$-lactamases are near the active sites of the respective enzymes, but others are clearly outside of these regions (3). Of this latter type of mutations, those at positions 164 and 179 are 10–12 Å from Ser$^{70}$, the active site residue that is acylated in the course of turnover chemistry. Although the effects of these amino acid substitutions are indirect, they nonetheless can result in profound phenotypic consequences.

In many cases, expanded-spectrum $\beta$-lactams survive in bacterial strains with class A $\beta$-lactamases because of the poor affinity of the enzymes for these substrates. The structural basis for this poor affinity appears to be the steric bulk of the drugs’ 7β functionalities. In contrast, the size of the functionality does not interfere with the interactions of these drugs with the penicillin-binding proteins, which are related proteins to $\beta$-lactamases and are the targets of inhibition by these antibiotics, so the antibiotics retain their activity. Kinetic analyses of the TEM$_{\mu}$C19 $\beta$-lactamase with ceftazidime indicated that affinity of the enzyme for this antibiotic is extremely poor. However, at high concentrations of ceftazidime in vitro, the enzyme is ultimately modified and subsequently undergoes deacylation to complete the catalytic cycle. Kinetic measurements indicated that, whereas both the acylation and deacylation steps are slow processes, deacylation is slower, and therefore rate-limiting in the course of the turnover chemistry for this $\beta$-lactamase. With this information at hand, we modeled the acyl-enzyme intermediate in the active site of the TEM$_{\mu}$C19 $\beta$-lactamase. Since the deacylation process appears so slow, the elimination of the C$_3$ leaving group of ceftazidime occurs while the drug is still bound to the enzyme prior to deacylation. Therefore, the relevant structure for modeling was species C (Scheme 1). The energy-minimized model of this
species in the active site of the TEM\textsubscript{pUC19} \(\beta\)-lactamase indicated that indeed there are severe steric clashes between the 7\(\beta\) functionality of ceftazidime and the \(\Omega\)-loop that spans residues 164–179 in the active site. We have reported earlier the model for the acyl-enzyme intermediate of the same enzyme class (16). As for the model with ceftazidime (see above), the model for cefepime showed a steric clash between its 7\(\beta\) functionality and the \(\Omega\)-loop; in each case, therefore, such interactions are responsible for the relatively poor affinity of the enzyme for these expanded-spectrum \(\beta\)-lactamases.

The \(\Omega\)-loop appears to have evolved to serve as a structural template for Glu\textsuperscript{166} of the class A \(\beta\)-lactamases (17). Glu\textsuperscript{166} is the only residue that is universally conserved in these enzymes among the residues of the \(\Omega\)-loop (164–179). All other residues within this secondary structural element are variable within the enzyme class (17). There seems to be a consensus opinion that the function of Glu\textsuperscript{166} is to serve as a general base to activate the enzyme for the deacylation step in the turnover of different types of substrates. Therefore, an unfavorable interaction of a substrate such as ceftazidime with the \(\Omega\)-loop could conceivably affect the deacylation step for turnover, an assertion that our kinetics data support.

If the steric encumbrance at the \(\Omega\)-loop is reduced by mutation of the parental \(\beta\)-lactamase, the restriction for turnover of the expanded-spectrum \(\beta\)-lactams might thereby be relaxed in the resulting variant. Indeed, nature has done just that by selection of mutants with amino acid substitutions at position 164. The amino acid at this position is arginine in the TEM\textsubscript{pUC19} \(\beta\)-lactamase, but some mutants that confer resistance to expanded-spectrum \(\beta\)-lactam have replacements of Arg\textsuperscript{164} with amino acids that have smaller side chains, such as serine or histidine (4). The arginine side chain essentially fills the interior of the \(\Omega\)-loop (Fig. 2), but in these mutant variants the shorter side chains would permit the collapse of the \(\Omega\)-loop to fill the “void.” If this were to happen, the resulting enlargement of the active site would permit better accommodation of substrates with a bulky 7\(\beta\) functionality. Our kinetics data indicate that this is the case, as the affinity for the expanded-spectrum cephalosporins increases for these mutant enzymes. As a matter of fact, microscopic rate constants (Table III) clearly indicate that an improved affinity with the enzymes mutated at position 164 is accompanied by enhancement of the microscopic rate constants for both acylation and deacylation in the course of turnover of the expanded-spectrum cephalosporins. On the other hand, it is interesting to note that, whereas the Asp\textsuperscript{179} \(\rightarrow\) Gly and Asp\textsuperscript{179} \(\rightarrow\) Asn mutations improved the affinity of the resultant enzymes for the extended-spectrum cephalosporins (lowered \(K_s\) values), these enzymes generally have reduced rates of acylation and deacylation (Table III). Thus, it would appear that in some cases, as in the mutants with replacements of Arg\textsuperscript{164}, enhancement of the microscopic rate constants may contribute to the expanded-spectrum phenotype, together with improved affinity of the enzyme for the substrates. The improved affinity merely allows the enzyme to reach saturation more readily. The facility of the subsequent bond-making and bond-breaking steps are important intuitively, but these steps may not proceed effectively unless the affinity for the substrate is raised sufficiently. However, because the mutants with glycine or asparagine at position 179 do not meet both criteria, they are not as effective as those with serine or asparagine at position 164. It is interesting to note that mutations at position 179 for the TEM enzyme have not yet been reported in clinical isolates. However, two mutations at position 179 for the SHV enzyme have been reported (36, 37).

We have described a multidisciplinary effort in elucidating the mechanistic and structural bases that underlie the properties of the extended-spectrum TEM \(\beta\)-lactamase mutants with amino acid substitutions at positions 164 and 179. With the emergence of mutant \(\beta\)-lactamases in clinical isolates, these enzymes have been the subject of intense investigation, and they serve as versatile systems for the study of evolution of function in biological systems. It is hoped that detailed knowl-
edge that emerges from such studies may help to understand
the underlying factors in evolution of novel enzyme variants,
and also facilitate strategies for development and use of β-lac-
tam antibiotics.

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