Selection and Characterization of Amino Acid Substitutions at Residues 237–240 of TEM-1 β-Lactamase with Altered Substrate Specificity for Aztreonam and Ceftazidime

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Recently, natural variants of TEM-1 β-lactamase with amino acid substitutions at residues 237–240 have been identified that have increased hydrolytic activity for extended-spectrum antibiotics such as ceftazidime. To identify the sequence requirements in this region for a given antibiotic, a random library was constructed that contained all possible amino acid combinations for the 3-residue region 237–240 (ABL numbering system) of TEM-1 β-lactamase. An antibiotic disc diffusion method was used to select mutants with wild-type level activity or greater for the extended-spectrum cephalosporin ceftazidime and the monobactam aztreonam. Mutants that were selected for optimal ceftazidime hydrolysis contained a conserved Ala at position 237, a Ser for Gly substitution at position 238, and a Lys for Glu at position 240. Mutants selected for aztreonam hydrolysis exhibited a Gly for Ala substitution at position 237, a Ser for Gly substitution at position 238, and a Lys/Arg for Glu at position 240. The role of the A237G substitution in differentiating between ceftazidime and aztreonam was further investigated by kinetic analysis of the A237G, E240K, G238S:E240K, and A237G:G238S:E240K enzymes. The A237G single mutant and the G238S:E240K double mutant exhibited increased in catalytic efficiency for both ceftazidime and aztreonam. However, the triple mutant A237G:G238S:E240K displayed a 12-fold decrease in catalytic efficiency for ceftazidime but a 3-fold increase for aztreonam relative to the G238S:E240K double mutant. Thus, the A237G substitution increases ceftazidime hydrolysis when present alone but antagonizes ceftazidime hydrolysis when it is combined with the G238S:E240K substitutions. In contrast, the A237G substitution acts additively with the G238S:E240K substitutions to increase aztreonam hydrolysis.

The most common mechanism of bacterial resistance to β-lactam antibiotics is the production of β-lactamase, a bacterial enzyme that catalyzes the hydrolysis of β-lactams. β-Lactamases are grouped into 4 classes (A, B, C, and D) based on primary sequence. TEM-1 β-lactamase, a class A serine hydrolase, is the most prevalent β-lactamase found in Gram-negative bacteria. TEM-1 β-lactamase is capable of hydrolyzing both penicillins and cephalosporins. However, it cannot hydrolyze the recently developed extended-spectrum antibiotics, such as ceftazidime and aztreonam. Extended-spectrum antibiotics were developed in part to combat Gram-negative bacteria that had developed resistance to existing penicillins and first and second generation cephalosporins through the expression of β-lactamases such as TEM-1. However, within a few years after the introduction of the extended-spectrum antibiotics, clinical isolates were discovered that were capable of hydrolyzing these new antibiotics (1). These enzymes, termed extended-spectrum β-lactamases, contain one to four amino acid substitutions near the active site and are derived either from the TEM or SHV β-lactamases (2). These substitutions occur at residues 104, 164, and 237–240, both as individual and as combinatorial mutations (numbering of amino acids according to Ambler et al. (26)). Specifically, the substitutions of a lysine for glutamate at position 104 (E104K), a serine for arginine at position 164 (R164S), a serine for a glycine at position 238 (G238S), and a lysine for a glutamate at position 240 (E240K), either alone or in combination, have been shown to provide increased catalytic activity toward the extended-spectrum cephalosporin ceftazidime (3–6). Understanding how amino acid substitutions alter the substrate specificity of β-lactamase for a given antibiotic may aid in the design of new antibiotics that avoid inactivation by extended spectrum β-lactamases. For example, by knowing if amino acid substitutions in extended spectrum β-lactamases act through enhanced binding of a specific part of an antibiotic side chain it may be possible to modify that part of the antibiotic to avoid inactivation. In addition, knowledge of how mutations alter the substrate specificity of β-lactamase may be of direct empiric value for combination therapy. If the set of substitutions in β-lactamase that result in increased activity are different for two antibiotics then the combination of those antibiotics in therapy may avoid the development of resistance.

Previously, random replacement mutagenesis has been used to randomize the nucleotide sequence of three contiguous codons in the blaTEM-1 gene to create libraries that encode all possible amino acid combinations for the target region (6–10). In the random library covering residues 238–241, β-lactamase mutants were selected and characterized having 100-fold higher activity than the wild-type level toward ceftazidime (6). That study determined that the G238S and E240K substitutions were the primary cause for ceftazidime resistance while substitutions at position 241 provided only a minor improvement.

While residue 241 is on the outer edge of the active site, crystallographic studies of TEM β-lactamase have shown residue 237 to be part of the hydrogen-bond network that stabilizes initial binding of the substrate in the active site (11, 12). Since residues 237–240 are the only contiguous residues in which substitutions have been shown to greatly increase the hydrol-
ysis spectrum of TEM β-lactamase (4–6, 8, 13), and residue 237 seems to have a greater importance than residue 241 in hydrolysis, the region 237–240 is more relevant for the study of enzyme-substrate interactions in extended-spectrum β-lactamases. In this report, random replacement mutagenesis has been used to create the random library L237–240, which contains all possible amino acid combinations for residues 237–240. This library was used to select mutants with high levels of activity toward the cephalosporin ceftazidime and the monobactam aztreonam to study the importance of this region in determining substrate specificity. Cefazidime and aztreonam contain an identical aminothiazole-oxime side chain and so amino acid substitutions that affect ceftazidime hydrolysis may exhibit a similar effect on aztreonam hydrolysis. In fact, analysis of kinetic parameters of the E104K, R164S, G238S, and E240K enzymes indicates that the catalytic efficiency of each of these enzymes is increased for both cefazidime and aztreonam (4, 5). In this study, the majority of mutants had a serine for glycine substitution at position 238 and a lysine or arginine for glutamate substitution at position 240, which reinforces the importance of the G238S and E240K/R substitutions in the hydrolysis of antibiotics with an aminothiazole-oxime side chain (3–6). All of the mutants selected for ceftazidime hydrolysis had the wild-type alanine conserved at position 237, but 17 out of 18 mutants selected for aztreonam hydrolysis had an A237G substitution. The role of the individual substitutions was determined by introducing the substitutions individually and in combination. Both the A237G single and G238S:E240K double substitution improve catalytic efficiency for both cefazidime and aztreonam. However, when the A237G substitution is added to the double mutant, catalytic efficiency is decreased 12-fold for ceftazidime but is increased 3-fold for aztreonam. Thus, the effect of the A237G substitution on ceftazidime hydrolysis is strongly dependent on whether it resides in the wild-type or G238S:E240K enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—All enzymes were purchased from New England Biolabs, except T7 DNA polymerase, which was purchased from U. S. Biochemical Corp. Cefazidime was provided by Glaxo (Greenford, United Kingdom) and aztreonam was provided by Bristol-Myers Squibb, Inc. Nitrocefin and antibiotic paper discs containing either cefazidime or aztreonam were purchased from Becton Dickinson (Cockeysville, MD). G-75 Sephadex was obtained from Pharmacia Biotech Inc. (Piscataway, N.J.).

*Escherichia coli* Strains and Plasmids—*E. coli* BW231 [F- lysA61–62, Δ(lacI*)lacZΔM15 proAB, Δ(lac prophage)], XL1-Blue and XL1-B are derivatives of *E. coli* K12 and carry a temperature-sensitive lacZ allele. XL1-Blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, F′ [λpir::Tn10Tet’proAB Δ(lacZΔM15)] lacZΔM15) was used for the propagation of plasmid DNA prior to mutagenesis (14). *E. coli* ES139 [lacZΔM15, mutS201]; Φ36, thyA36, rha5, metB1, deoC1] (rrnD-rrnE) was used for the introduction of mutated DNA (15). *E. coli* XL1-Blue [recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, F′ [λpir::Tn10Tet’proAB Δ(lacZΔM15)] lacZΔM15] was used for the determination of antibiotic susceptibility and the preparation of single stranded DNA (16).

Plasmid pBG66 is the parent plasmid of all random library constructions (9). The pBG66 plasmid is a 4.8-kilobase derivative of pBR322 and pBR325 that contains the wild-type *bla*<sub>TEM</sub>-<sub>R</sub> gene and the cat gene, which encodes for chloramphenicol acetyltransferase. The plasmid also contains the ColE1 and fl origins of DNA replication.

**Oligonucleotides and Random Replacement Mutagenesis—Oligonucleotide primers for mutagenesis were synthesized by Genentech, Inc., at the PAN facility at Stanford University Medical School, and at Genosys Biotechnologies, Inc. Random replacement mutagenesis was done as described previously (17).

Briefly, the strategy behind the modified mutagenesis was to first insert a unique XhoI restriction site into a location within the *bla* gene, which has been targeted for mutagenesis. The XhoI recognition sequence is flanked by 12 base arms which are complementary to the sequence adjacent to the site targeted for mutagenesis. The restriction site was positioned at or near the middle of the site to be randomized, and the second base of the middle codon was deleted to create a frameshift mutation. A *XhoI* restriction site was previously inserted at codon 238 (9). The frameshift mutation, resulting from this insertion, renders the *bla* gene non-functional. Subsequent randomization is achieved by replacing the unique restriction site with a 9-base randomized DNA sequence. An oligonucleotide was designed to replace the 9-base window (including the XhoI site) with sequence 5′-NNS NNS NNS-3′ (where N indicates an equal probability of any base, and S indicates an equal probability of either C or G). This insured all amino acids would be sampled in the window. Two 14-base complementary arms flank the random sequence. The calculations involved in the determination of the percent of randomization have been described previously (9). Library DNA was electroporated into *E. coli* XL1-B cells for further screening.

**Plasmid Isolation and DNA Sequencing—** Single-stranded plasmid DNA was prepared for sequencing as described (18). DNA sequencing was performed using the dideoxy chain termination method (19). Oligonucleotides were designed to prime synthesis from specific sites within the *bla*<sub>TEM</sub>-<sub>R</sub> gene.

**Selection of Active Mutants—** Selections were done using the disc diffusion method. The TEM-1 β-lactamase or the L237–240 was introduced into *E. coli* XLI-1B and was grown in 2 × YT media plus 12.5 μg/ml chloramphenicol to stationary phase with aeration at 37°C. A 1:20 dilution of this culture was made with Mueller-Hinton media, and a sterile cotton swab was used to plate the dilution on Mueller-Hinton agar plates. Antibiotic papers containing either 30 μg of cefazidime or aztreonam were applied to the surface of the plates which were then incubated overnight at 37°C. The zone of inhibition for wild-type β-lactamase was used as a control. Any colonies from L237–240 that grew near or within the wild-type zone of inhibition were considered to have greater than or equal to TEM-1 activity.

**Antibiotic Susceptibility—** Minimum inhibitory concentrations (MICs) were determined by broth microdilution. 1 × 10<sup>8</sup> *E. coli* XLI-B cells containing the selected mutant β-lactamase were inoculated into microtiter wells containing 100 μl of LB media having 2-fold dilutions of the antibiotic being tested. The ranges of antibiotic concentrations tested for cefazidime and aztreonam were 0.03–64 μg/ml. The microtiter plates were incubated at 37°C for 18–24 h. The plates were examined visually, and the lowest concentration of antibiotic that inhibited visual growth was scored as the MIC. A 2-fold difference in MIC values was determined to be insignificant.

**Site-directed Mutagenesis—** The E240K and G238S:E240K mutants were previously constructed by cassette mutagenesis (6). The A237G mutant was originally isolated from the L237–240 random library in a selection for the presence of a frameshift mutation (9). The β-Lactamase Purification—TEM-1 β-lactamase and the mutant β-lactamases were purified to >90% homogeneity. The wild-type TEM-1, E240K, and G238S:E240K enzymes were previously cloned into an expression vector under the control of the tac promoter (6). *E. coli* XLI-1-Blue cells containing the expression vector were grown in LB media (18) containing 25 μg/ml kanamycin to early log phase. An OD<sub>600</sub> of 0.5, 0.1 μg/ml isopropyl-1-thio-o-galactopyranoside was added and the culture was incubated an additional 3 h until it reached late log phase. In contrast, the A237G and A237G;G238S:E240K enzymes were purified from *E. coli* XLI-1-B cells containing the plasmid pBG66 plasmid and were expressed from the natural β-lactamase promoter. These strains were grown in culture for 9 h to reach late log phase. From this point, all enzymes were purified by the method that follows. β-Lactamase and other periplasmic proteins were first isolated by an osmotic shock procedure (20). The solution obtained by osmotic shock was adjusted to a final concentrate of 100 mM NaAc, pH 7.5, 800 mM NaCl (buffer A). The protein solution was concentrated to a 5-ml volume with an Amicon Centriprep-10 concentrator. The concentrated protein solution was then applied to a 1-ml HiTrap zinc chelating column (Pharmacia). β-Lactamase bound strongly to the column while other periplasmic proteins eluted out with buffer A. β-Lactamase was eluted using a linear gradient of buffer B (100 mM NaAc, pH 4.0, 800 mM NaCl). Fractions containing β-lactamase activity were identified by nitrocefin hydrolysis and SDS-polyacrylamide gel electrophoresis. The β-lactamase fractions were concentrated with a Centriprep-10 concentrator. The β-lactamase was further purified by Sephacryl G-75 gel filtration chromatography using a 25 mM sodium phosphate buffer, pH 7.0. The purity of the enzymes was verified by SDS-polyacrylamide gel electrophoresis. All preparations were >90% pure. The purified enzymes were stored at 4°C until the determination of kinetic parameters.

**Enzyme Kinetics—** The kinetics of TEM-1 β-lactamase and the sequence. 

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1 The abbreviations used are: L237–240, the random library containing all possible amino acid combinations for residues 237–240; MIC, minimum inhibitory concentration.
lected mutants were determined with ampicillin, cephalexin, ceftazidime, and aztreonam. The extinction coefficients used were: ampicillin, 235 nm, Δε = 900 M⁻¹ cm⁻¹ (6); cephalexin, 260 nm, Δε = 10,200 M⁻¹ cm⁻¹ (21); ceftazidime, 260 nm, Δε = 8,660 M⁻¹ cm⁻¹ (22); aztreonam, 318 nm, Δε = 660 M⁻¹ cm⁻¹ (22). The hydrolysis of the substrates was monitored spectrophotometrically at 30°C in 0.05 M phosphate buffer, pH 7.0, on a Beckman model DU 640 spectrophotometer using a 0.1- or 1.0-cm pathlength cuvette as needed. Kinetic parameters Vₘₐₓ and Kᵣ were determined by 1) initial velocity kinetic analysis by fitting to the equation of a rectangular hyperbola using unweighted nonlinear least squares (23), or 2) using complete progress curves with starting concentrations at least 3 ≫ Kᵣ. The progress curves were evaluated by determining the instantaneous rate at a minimum of 50 points in the reaction by taking the derivative of the substrate concentration at those points in the reaction course. This data was fitted to the Michaelis-Menten equation using unweighted nonlinear least squares (23). To insure that substrate inhibition was not occurring, initial rates were determined at 6 to 8 substrate concentrations chosen to bracket the Kᵣ value determined by the progress curve. In the case of aztreonam, the Kᵣ was measured as the Kᵣ with cephalexin as the reporter substrate. The enzyme and inhibitor were preincubated for 5 min at 30°C before the substrate was added. At least five concentrations of reporter substrate bracketing the Kᵣ were tested with at least three concentrations of inhibitor. Enzyme concentration was at least 10-fold less than substrate concentrations. Both were done using the extended-spectrum cephalosporin ceftazidime on velocity measurements at 10, 15, and 20°C. A 3-Å hydrogen bond between the lactam carbonyl oxygen and the main chain amide group of Ser-64 (Ser-70 in the TEM-1 enzyme) and a 2.9-Å hydrogen bond between the lactam carbonyl oxygen and the main chain amide group of Ser-318 (Ala-237 in TEM-1 enzyme). The 1.7-Å crystal structure of benzylpenicillin with a E166N TEM-1 mutant (11) describes the acyl-enzyme complex as having a 2.7-Å hydrogen bond between the lactam carbonyl oxygen and the Ser-70 main chain amide group and a 3.0-Å hydrogen bond between the lactam carbonyl oxygen and the Ala-237 main chain amide group. The aztreonam molecule was manually docked into the active site of the TEM-1 enzyme using Quanta, version 3.3, from Molecular Simulations, Inc. (Waltham, MA). The substrate was fitted to position the β-lactam carbonyl oxygen in the oxyanion hole within ±0.5 Å of the distances mentioned above. The model was adjusted manually to relieve obvious steric problems with the enzyme. No attempt was made to alter the conformation of the aztreonam molecule or to optimize the fit by energy minimization.

RESULTS

Selection of Mutants from the 237–240 Library—Naturally occurring amino acid substitutions at positions 237, 238, and 240 have been described in several extended-spectrum β-lactamases. For example, TEM-3, TEM-4, TEM-8, TEM-14, TEM-15, and TEM-19 contain the G238S substitution, TEM-10 and TEM-24 contain the E240K substitution, and TEM-5 contains the A237T:E240K double substitution (2). Note that residues 238 and 240 are adjacent to each other in the TEM sequences. The absence of residue 239 is due to the ABL numbering system for Class A β-lactamases (26). In previous experiments with the random library 238–241, β-lactamase mutants were selected that had 100-fold greater resistance than wild-type toward ceftazidime (6). It was found that the multiple substitution of G238S:E240K was responsible for the large increase in catalytic efficiency for ceftazidime.

In order to better study the role of substitutions at positions important for extended-spectrum β-lactamase activity, the random library 237–240 was constructed. Selection experiments were done using the extended-spectrum cephalexin ceftazidime and the monobactam aztreonam (Fig. 1). The similarity of these substrates should provide further information about the ability of substitutions in this region to not only increase cat-
Altered Substrate Specificity of β-Lactamase

Table: MICs (μg/ml)

| Substrate | CAZ | ATM |
|-----------|-----|-----|
| 237       | 0.5 | 0.12|
| 238       | 32  | 32  |
| 240       | 32  | 16  |

A) CAZ selected mutants

| Mutant | A | G | E |
|--------|---|---|---|
| 5      | A | S | R |
| 10     | A | S | R |
| 5      | A | N | K |

B) ATM selected mutants

| Mutant | G | S | R |
|--------|---|---|---|
| 6      | G | S | R |
| 3      | G | L | R |
| 2      | G | S | Q |
| 2      | G | T | R |
| 3      | G | T | K |
| 2      | G | N | T |
| 1      | A | S | R |

Fig. 2. Amino acid sequences of mutants selected from the L237–240 random library. The number of times each sequence occurred is indicated to the left of each sequence. A, mutants selected with ceftazidime. B, mutants selected with aztreonam.

Alterations in substrate specificity can be due to the mutations conferred by hydrolytic activity, the MICs were determined for aztreonam and ceftazidime for all the selected mutants (Fig. 2). The MICs for ceftazidime were 60- to 100-fold greater than wild-type for mutants with the G238S and E240K/R substitutions. However, the G238S:E240K/R mutants that contained the additional substitution of A237G had MICs only 30-fold greater than wild-type. Thus, the addition of the A237G substitution to the G238S:E240K double mutant decreased the activity of the enzyme toward ceftazidime.

Fig. 2

Antibiotic Susceptibility—To better understand the extent to which the substitutions conferred increased hydrolytic activity, the MICs were determined for aztreonam and ceftazidime for all the selected mutants (Fig. 2). The MICs for ceftazidime were 60- to 100-fold greater than wild-type for mutants with the G238S and E240K/R substitutions. However, the G238S:E240K/R mutants that contained the additional substitution of A237G had MICs only 30-fold greater than wild-type. Thus, the addition of the A237G substitution to the G238S:E240K double mutant decreased the activity of the enzyme toward ceftazidime.

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The MICs for aztreonam were 250-fold higher than wild-type for the G238S:E240K double mutant. However, in contrast to the ceftazidime MICs, the addition of the A237G substitution to the double mutant resulted in a further increase in the aztreonam MIC. These results suggest that the A237G substitution allows the enzyme to discriminate between aztreonam and ceftazidime.

Kinetic Analysis—In order to further understand the effects of the substitutions described above on catalytic activity, the A237G, E240K, G238S:E240K, and A237G:G238S:E240K β-lactamases were purified for kinetic analysis (“Experimental Procedures”). The A237G, E240K, and G238S:E240K mutants were constructed previously (6, 9). The kinetic parameters for ampicillin, cephaloridine, ceftazidime, and aztreonam hydrolysis were determined for the wild-type TEM-1, A237G, E240K, G238S:E240K, and A237G:G238S:E240K enzymes. The structures of these antibiotics are shown in Fig. 1.

The kinetics of ampicillin and cephaloridine hydrolysis (Tables I and II) were determined to compare the effect of the substitutions on the hydrolysis of a penicillin and a non-extended-spectrum cephalosporin with that of ceftazidime and aztreonam. The effect of the A237G substitution on the hydrolysis of ampicillin and cephaloridine followed a similar pattern in that for both substrates the $K_m$ value was increased relative to the wild-type enzyme. The double mutant G238S:E240K exhibits a large decrease in both $k_{cat}$ and $K_m$ values. This has been reported previously and it was shown that the majority of this effect is due to G238S substitution (6). The $K_m$ values determined for the G238S:E240K enzyme in this study are somewhat lower than has been described previously (6). However, all data reported here was done with the same batches of enzyme and antibiotic substrate to facilitate a direct comparison of values. The addition of the A237G substitution to the G238S:E240K enzyme increases the $K_m$ value of the triple mutant for both ampicillin and cephaloridine relative to the G238S:E240K double mutant. Therefore, both alone and in combination with the G238S:E240K substitutions, the A237G substitution increases the $K_m$ value for ampicillin and cephaloridine hydrolysis.

As seen in Table III, all of the mutant enzymes had increased catalytic efficiency for ceftazidime. With ceftazidime as substrate, a high $K_m$ value (>1000 μM) prevented the individual values of $k_{cat}$ and $K_m$ from being determined for all but the G238S:E240K enzyme. The $k_{cat}/K_m$ values were estimated by examining the reaction rate at [S] ≪ $K_m$ (24). As described previously (6), the effects of the G238S and E240K substitutions on ceftazidime hydrolysis were that G238S causes a significant decrease in $K_m$, allowing the $k_{cat}$ and $K_m$ of G238S:
E240K to be measured, and E240K causes an increase in \( k_{\text{cat}} \) and a small decrease in \( K_m \) (6). Accordingly, the double mutant G238S:E240K provides a 1294-fold increased catalytic efficiency (\( k_{\text{cat}}/K_m \)) over the wild-type enzyme. Interestingly, the A237G substitution alone provides a 13-fold increase in catalytic efficiency for ceftazidime, but the triple mutant A237G:G238S:E240K provides only a 109-fold increase, which is 12-fold less than the double mutant. The results suggest that the A237G substitution, while beneficial on its own, detracts from the contributions of the G238S:E240K double mutant. Because the \( K_m \) of the G238S:E240K double mutant is 178 \( \mu \)M and the \( K_m \) of the A237G:G238S:E240K triple mutant is too high to measure (\( >1000 \mu \)M), it can be concluded that the addition of the A237G substitution to the double mutant results in at least a 5-fold increase in \( K_m \). Note that an increase in \( K_m \) is also observed with ampicillin and cephaloridine as substrates (Tables I and II). However, because of the inability to measure \( K_m \) in the A237G single mutant, it is unclear whether the increase in catalytic efficiency of this enzyme is due to decreased \( K_m \) or increased \( k_{\text{cat}} \) or both.

With aztreonam as the substrate (Table IV), each substitution lowered the \( K_m \) value such that \( k_{\text{cat}} \) and \( K_m \) could be determined for each enzyme except wild-type. The effect of the A237G and E240K substitutions was to lower the \( K_m \) to a level that we could measure. An even larger reduction, relative to that of A237G and E240K, was observed for the \( K_m \) values of G238S:E240K and A237G:G238S:E240K, with reductions of an additional 200–600-fold. The addition of the G238S substitution to the E240K enzyme had the effect of further reducing both \( K_m \) and \( k_{\text{cat}} \). In contrast to the kinetic patterns observed with ceftazidime, the triple mutant A237G:G238S:E240K had the highest catalytic efficiency for aztreonam, with a 3437-fold increase over the wild-type enzyme. The reason for this is that the addition of the A237G substitution to the G238S:E240K enzyme has the effect of lowering \( K_m \) and thus providing a 3-fold increase in catalytic efficiency over the G238S:E240K double mutant. It is interesting that the A237G substitution resulted in a lower \( K_m \) value when aztreonam was the substrate versus a higher \( K_m \) value for ampicillin, cephaloridine, and ceftazidime.

A comparison of the substrate specificity, as measured by the catalytic efficiency, of wild-type TEM-1 \( \beta \)-lactamase and each of the mutant enzymes is shown in Fig. 3. It is apparent from the data that the wild-type enzyme has very high catalytic efficiency for ampicillin and cephaloridine and poor catalytic efficiency for ceftazidime and aztreonam. The double mutant G238S:E240K, although, has moderately high catalytic efficiency for all substrates. With the addition of A237G, however, the triple mutant exhibits a further increase in catalytic efficiency for aztreonam hydrolysis while showing reduced efficiency for ampicillin, cephaloridine, and ceftazidime hydrolysis. Thus, the double substitution of G238S:E240K provides the wild-type enzyme with the ability to hydrolyze antibiotics with an aminothiazole-oxime side group, and the further addition of A237G allows the enzyme to differentiate between a monobactam and a cephalosporin with the aminothiazole-oxime side group.

**DISCUSSION**

The goal of this study was to identify substitutions in the 237–240 region of TEM-1 \( \beta \)-lactamase that alter specificity and enhance catalytic efficiency toward different antibiotic substrates, such as the cephalosporin ceftazidime and the monobactam aztreonam. In order to accomplish this goal, random replacement mutagenesis was used to completely randomize residues 237–240 to form a random library that contains all the possible amino acid combinations for that region. Then, mutants were selected that had greater than the wild-type

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### Table I

**Kinetic parameters of wild-type (wt) and mutant \( \beta \)-lactamases for ampicillin**

| \( \beta \)-Lactamases | \( k_{\text{cat}} \) | \( K_m \) | \( k_{\text{cat}}/K_m \) | Relative* |
|------------------------|-----------------|---------|-------------------|---------|
| wt 237-AGE-240         | 1625 ± 74       | 63 ± 9  | 2.58 \( \times \) 10^7 | 1.00    |
| A237G                  | 1530 ± 94       | 100 ± 13 | 1.53 \( \times \) 10^7 | 0.59    |
| E240K                  | 851 ± 96        | 72 ± 19 | 1.18 \( \times \) 10^7 | 0.46    |
| G238S:E240K            | 8.3 ± 0.5       | 3.7 ± 1.1 | 2.24 \( \times \) 10^6 | 0.09    |
| A237G:G238S:E240K      | 24 ± 1          | 28 ± 2  | 8.57 \( \times \) 10^5 | 0.03    |

* Ratio of \( k_{\text{cat}}/K_m \) of the mutant relative to the wild-type.

### Table II

**Kinetic parameters of wild-type (wt) and mutant \( \beta \)-lactamases for cephaloridine**

| \( \beta \)-Lactamases | \( k_{\text{cat}} \) | \( K_m \) | \( k_{\text{cat}}/K_m \) | Relative* |
|------------------------|-----------------|---------|-------------------|---------|
| wt 237-AGE-240         | 1070 ± 124      | 761 ± 44 | 1.41 \( \times \) 10^6 | 1.00    |
| A237G                  | 849 ± 38        | 1635 ± 94 | 5.19 \( \times \) 10^5 | 0.37    |
| E240K                  | 281 ± 22        | 669 ± 95 | 4.20 \( \times \) 10^5 | 0.30    |
| G238S:E240K            | 9.7 ± 0.4       | 16.1 ± 2.0 | 6.02 \( \times \) 10^4 | 0.43    |
| A237G:G238S:E240K      | 10.3 ± 0.5      | 28.6 ± 3.0 | 3.60 \( \times \) 10^3 | 0.26    |

* Ratio of \( k_{\text{cat}}/K_m \) of the mutant relative to the wild-type.

### Table III

**Kinetic parameters of wild-type (wt) and mutant \( \beta \)-lactamases for ceftazidime**

| \( \beta \)-Lactamases | \( k_{\text{cat}} \) | \( K_m \) | \( k_{\text{cat}}/K_m \) | Relative* |
|------------------------|-----------------|---------|-------------------|---------|
| wt 237-AGE-240         | NM*             | NM      | 5.51 \( \times \) 10^4 | 1       |
| A237G                  | NM              | NM      | 6.89 \( \times \) 10^4 | 13      |
| E240K                  | 10 ± 2          | 5.64 \( \times \) 10^4 | 10      |
| G238S:E240K            | 12.7 ± 0.4      | 178 ± 10 | 7.13 \( \times \) 10^4 | 1294    |
| A237G:G238S:E240K      | NM              | NM      | 6.02 \( \times \) 10^3 | 109     |

* Ratio of \( k_{\text{cat}}/K_m \) of the mutant relative to the wild-type.

* NM, not measurable.
level of activity toward the given antibiotic. It was found that the preferred sequence for ceftazidime hydrolysis was a conserved alanine at position 237, a serine or asparagine at position 238, and a lysine or arginine at position 240. The preferred sequence for aztreonam hydrolysis involved more variability and was found to have a glycine at position 237, a serine, threonine, or asparagine at position 238, and a lysine, arginine, or threonine at position 240. Previous studies with TEM-1 and SHV-type \( \beta \)-lactamases have shown the importance of the G238S:E240K double mutant for ceftazidime hydrolysis (3, 6). For TEM-1 \( \beta \)-lactamase, the G238S:E240K double substitution has been shown to increase \( k_{\text{cat}} \) while decreasing \( K_m \) (6). Characterization of these enzymes for aztreonam hydrolysis indicate that the increases in \( k_{\text{cat}}/K_m \) are mostly due to very large reductions in \( K_m \).

To better understand the role of the A237G substitution, we determined the contributions of the A237G, E240K, G238S:E240K, and A237G:G238S:E240K substitutions in ampicillin, cephaloridine, ceftazidime, and aztreonam hydrolysis. Characterization of these enzymes for ampicillin and cephaloridine hydrolysis indicate that the A237G substitution results in an increased \( K_m \) value which causes a slight decrease in the overall catalytic efficiency for these substrates (Tables I and II). However, characterization of these enzymes for ceftazidime hydrolysis indicate that, although A237G alone provides an increase in catalytic efficiency, when it is added to the G238S:E240K double mutant a reduction in catalytic efficiency occurs and therefore the A237G substitution detracts from the contributions of the other substitutions.

In contrast to the hydrolysis of ceftazidime, the A237G substitution is beneficial for aztreonam hydrolysis both individually and in combination with the G238S:E240K double mutant. The \( k_{\text{cat}}/K_m \) of the A237G mutant is increased 19-fold more than the wild-type enzyme (Table IV). In addition, the \( k_{\text{cat}}/K_m \) of the G238S:E240K double mutant for aztreonam hydrolysis is increased 3-fold when the A237G substitution is added (Table IV). Whereas optimal ceftazidime hydrolysis is achieved by the double mutant G238S:E240K, optimal aztreonam hydrolysis is achieved by the triple mutant A237G:G238S:E240K.

The contributions of the substitutions to the overall catalytic efficiency can be calculated for each substrate from the data in Tables I through IV. This was done using the following equation (24):

\[
\Delta G = -RT \ln \left( \frac{k_{\text{cat}}/K_m\text{mutant}}{k_{\text{cat}}/K_m\text{wild-type}} \right)
\]

which calculates the difference in binding energy between the wild-type and mutant enzymes in going from free enzyme and substrate to the transition state. With ceftazidime as the substrate, the A237G:G238S:E240K double mutant decreases the free energy barrier by 2.8 kcal/mol. The A237G and G238S:E240K substitutions decrease the free energy barrier by 1.5 and 4.3 kcal/mol, respectively. The free energy changes of these two mutants can be related to the triple mutant A237G:G238S:E240K by the following equation (27, 28):

\[
\Delta G_{\text{A237G:G238S:E240K}} = \Delta G_{\text{A237G}} + \Delta G_{\text{G238S:E240K}} + \Delta G_1
\]
Altered Substrate Specificity of β-Lactamase

The $\Delta G_1$ term represents a change in the interaction energy provided by the individual mutants to the triple mutant. If the contributions of the substitutions display simple additivity, the value of $\Delta G_1$ will be near zero. The value of $\Delta G_1$ for the triple mutant A237G-G238S-E240K is +3.0 kcal/mol. This value indicates complex additivity and that the contribution of the A237G substitution to transition state stabilization is lost when the substitution is added to the G238S-E240K double mutant. In fact, the A237G substitution antagonizes the transition state stabilization provided by the G238S-E240K substitutions.

Applying the same analysis to data from Table IV with aztreonam as substrate, $\Delta G = -4.9$ kcal/mol for the triple mutant A237G-G238S:E240K, while the values for A237G and G238S:E240K were $-1.8$ and $-4.3$ kcal/mol, respectively. The value of $\Delta G_1$ was +1.2 kcal/mol for the triple mutant. Although this value is non-zero, interaction energies from 1.0 to 1.5 kcal/mol are generally considered additive because of errors involved in calculating the free energy effect in the multiple mutant from the component mutants (28, 29). Thus, the addition of A237G to G238S:E240K exhibits simple additivity. The $\Delta G$ for the A237G-G238S:E240K enzyme with ampicillin as substrate was +2.1 kcal/mol with a $\Delta G_1$ term of +0.3 kcal/mol, while the A237G-G238S:E240K $\Delta G$ for cephalexin was +0.8 kcal/mol with a $\Delta G_1$ term of -0.3 kcal/mol. Thus, for aztreonam the contribution of the A237G substitution to transition state stabilization is retained when the substitution is added to the G238S:E240K double mutant. Also, the destabilizing effect of the substitutions on ampicillin and cephalexin hydrolysis displays simple additivity, in contrast to the non-additive destabilizing effect seen with ceftazidime.

A review of site-directed mutagenesis studies of tyrosyl-tRNA synthetase and subtilisin BPN’ has shown that complex additivity, or non-additivity, in transition state stabilization is observed in two instances (28). First, when the mutation sites are very close together a change in the interaction energy between the sites often occurs. This is thought to be due to either a disruption in direct contacts by the residues or an indirect change in electrostatic interactions or structural perturbations of the residues. Second, when a mutation site is involved in a cooperative interaction in the enzyme mechanism a change in the rate-limiting step may occur. Thus, the complex additivity shown by the TEM-1 β-lactamase A237G and G238S:E240K mutants for ceftazidime hydrolysis is consistent with the above mentioned observations on the additivity relationships of adjacent residues (28). However, these same mutants display simple additivity for ampicillin, cephalexin, and aztreonam hydrolysis. There are several possible explanations for the non-additivity observed with ceftazidime but not the other antibiotics. For example, the A237G substitution may introduce a conformational change in the G238S:E240K enzyme structure or the solvent structure around the active-site that antagonizes the contributions of the individual substitutions. However, the change in enzyme or solvent structure would not affect the hydrolysis of the other antibiotics. An alternative explanation is that ceftazidime binding may introduce a conformational change in the G238S:E240K enzyme that does not occur in the A237G-G238S:E240K triple mutant. This model more readily explains why non-additivity is observed only with ceftazidime. Support for this model comes from x-ray crystallographic studies of a DD-peptidase with cefalothin or cefotaxime in the active site (30). It was found that a conformational change in an active-site threonine residue (T301) occurs in the cefotaxime-enzyme complex but not the cefalothin complex (30). Finally, the cefazidime substrate may undergo a conformational change upon binding the G238S:E240K enzyme that is impeded by the addition of the A237G substitution.

In the case of aztreonam hydrolysis, the A237G enzyme exhibits a large reduction in the $K_m$ value and when the A237G substitution is added to the G238S:E240K enzyme, the $K_m$ of this enzyme is further reduced. In contrast, the A237G substitution results in an increased $K_m$ for ampicillin and cephalexin hydrolysis both alone and in combination with the G238S:E240K substitutions. These opposite effects may be due to the presence of the single lactam ring in the monobactam compared to the fused ring system in the other antibiotics. The structure of an acyl-enzyme intermediate of TEM-1 β-lactamase with penicillin G (PenG) has been solved (11). This structure shows that the PenG carboxylate group at position C-3 of the thiazolidine ring forms strong hydrogen bonds to both the Arg-244 and Ser-235 side chains. An important difference between monobactams such as aztreonam and the penicillins and cephalexins is that the monobactams have a sulfonic acid group bonded directly to the nitrogen of the lactam ring in place...
of the functionally equivalent carboxylate group (Fig. 1). The sulfonic acid is therefore in a different position relative to the carbonyl-carbon of the lactam ring than is the carboxylate. This may result in weaker interactions between the sulfonic acid oxygens and the side chains of Ser-235 and Arg-244. A model of aztreonam in the active site of the wild-type TEM-1 β-lactamase is shown in Fig. 4. The A237G substitution may act by altering the conformational flexibility of the S3 β-strand on which both Ser-235 and Ala-237 are found. This could reposition the side chain of Arg-244 to rotate to a position closer to the sulfonic acid group. Alternatively, the removal of the methyl group at position 237 in the A237G mutant may allow the side chain of Arg-244 to rotate to a position closer to the sulfonic acid group and form a stronger hydrogen bond with the sulfonic acid group. Either of these possibilities might improve the binding of the substrate and account for the reduced Kₘ value.

A change in enzyme mechanism also must be considered. Acylation has been determined as the rate-limiting step of TEM-1 β-lactamase for the extended-spectrum cephalosporin ceftaxime, and the G238S substitution was shown to improve the acylation rate, although not enough to change the rate-limiting step (31). The large reduction in Kₘ for aztreonam could be explained by a possible change in rate-limiting step from acylation to deacylation. However, a detailed interpretation of the effect of the A237G substitution awaits further structural and kinetic data on the enzyme.

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Selection and Characterization of Amino Acid Substitutions at Residues 237-240 of TEM-1 β-Lactamase with Altered Substrate Specificity for Aztreonam and Ceftazidime

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