β-Lactamases inactivate β-lactam antibiotics by catalyzing the hydrolysis of the amide bond in the β-lactam ring. The plasmid-encoded class A TEM-1 β-lactamase is a commonly encountered β-lactamase. It is able to inactivate penicillins and cephalosporins but not extended-spectrum antibiotics. However, TEM-1-derived natural variants containing the G238S amino acid substitution display increased hydrolysis of extended-spectrum antibiotics. Two models have been proposed to explain the role of the G238S substitution in hydrolysis of extended-spectrum antibiotics. The first proposes a direct hydrogen bond of the Ser238 side chain to the oxime group of extended-spectrum antibiotics. The second proposes that steric conflict with surrounding residues, due to increased side chain volume, leads to a more accessible active site pocket. To assess the validity of each model, TEM-1 mutants with amino acids substitutions of Ala, Ser, Cys, Thr, Asn, and Val have been constructed. Kinetic analysis of these enzymes with penicillins and cephalosporins suggests that a hydrogen bond is necessary but not sufficient to achieve the hydrolytic activity of the G238S enzyme for the extended-spectrum antibiotics cefotaxime and ceftazidime. In addition, it appears that the new hydrogen bond interaction is to a site on the enzyme rather than directly to the extended-spectrum antibiotic. The data indicate that, for the G238S substitution, a combination of an optimal side chain volume and hydrogen bonding potential results in the most versatile and advantageous antibiotic hydrolytic spectrum for bacterial resistance to extended-spectrum antibiotics.

Bacterial resistance to β-lactam antibiotics, such as penicillins and cephalosporins, is primarily mediated by the production of β-lactamases, which catalyze the hydrolysis of β-lactam antibiotics to inactive products (1). TEM-1 β-lactamase is one of the most common plasmid-mediated β-lactamases in Gram-negative bacteria. It has a broad spectrum of hydrolytic activity that includes penicillins and cephalosporins, but not extended-spectrum antibiotics (2). Extended-spectrum cephalosporins were developed, in part, because of the ability of β-lactamases, such as TEM-1, to efficiently hydrolyze older penicillins and cephalosporins. These drugs, due to their larger side chains, were able to avoid inactivation by the TEM-1 enzyme. Unfortunately, after the introduction of extended-spectrum cephalosporins, clinical isolates were discovered containing TEM variants with active site mutations that allowed for hydrolysis of these antibiotics (3).

One particular amino acid substitution, a serine for glycine at position 238 in the active site, appears in natural variants of both the TEM and SHV (another common plasmid-encoded enzyme with 68% homology to TEM-1) enzymes and provides bacterial resistance to extended-spectrum cephalosporins. This substitution alone, or in combination with other substitutions, can provide clinically relevant levels of resistance (3). Two types of models have been proposed to explain the effectiveness of the G238S substitution in the hydrolysis of extended-spectrum cephalosporins (4–6). The first model involves a hydrogen bond between the serine residue and the oxime group of extended-spectrum cephalosporins (Fig. 1A) (5). This hydrogen bond would improve affinity of the enzyme for the antibiotic, and consequently improve the catalytic efficiency. The second model suggests that the serine residue, due to steric conflict with either residues 69 or 170, would improve affinity through an active site cavity expansion either by shifting the position of the B3 β-strand (Fig. 1B) (6) or shifting the position of an active site omega loop (Fig. 1C) (4), either of which would then allow extended-spectrum antibiotics more space to bind. Thus, the “steric conflict” models are also based on improving affinity for the antibiotic, but indirectly by altering the shape of the active site as opposed to the direct hydrogen bond interaction with substrate proposed in the first model.

To study the role of residue 238 and test the mechanisms proposed by the two types of models, a series of mutants (Ala, Ser, Cys, Thr, Asn, and Val) were constructed at position 238 with increasing side chain volume and alternating hydrogen bonding capabilities. The expectation for the first model is that only hydrogen bonding residues (Ser, Asn, and Thr) will provide an increase in the catalytic efficiency of extended-spectrum cephalosporins relative to the wild-type glycine residue. The expectation for the second model is that an increase in catalytic efficiency will follow an increase in side chain volume. Kinetic analysis of these enzymes shows that the hydrogen-bonding amino acids (Ser, Asn, and Thr) do provide an increase in hydrolysis of the extended-spectrum cephalosporins, with the G238S enzyme providing the largest increase. Interestingly, increases in catalytic efficiency observed with hydrogen-bonding amino acids are not limited to substrates containing a hydrogen bond acceptor, suggesting that an intramolecular hydrogen bond is formed to another site in the enzyme or to a structural water molecule. These results indicate that a hydrogen-bonding amino acid at position 238 is necessary but not sufficient to increase the hydrolysis of extended-spectrum cephalosporins to an extent that is clinically relevant (i.e. G238S). The Ala substitution also provides an increase in the...
hydrolysis of extended-spectrum cephalosporins relative to the wild-type enzyme. Therefore, the side chain volume of residue 238 does play a role in the hydrolysis of these antibiotics. From these results, it appears that only the Ser substitution combines a new intramolecular hydrogen bond interaction with an optimal increase in side chain volume resulting in a β-lactamase with significant levels of hydrolysis of extended-spectrum cephalosporins. The greater versatility of the Ser substitution over other amino acid substitutions in effecting these changes may explain why it is the only substitution seen at residue 238 in extended-spectrum β-lactamases.

**EXPERIMENTAL PROCEDURES**

**Materials**—All enzymes were purchased from New England Biolabs, except T7 DNA polymerase, which was purchased from U. S. Biochemical Corp. Ampicillin, benzylpenicillin, cephaloridine, cephalothin, and cefotaxime were purchased from Sigma. Cefazolin was provided by Glaxo. Nitrocefin was purchased from Becton Dickinson. G-75 Sephadex was obtained from Amersham Pharmacia Biotech.

*Escherichia coli* Strains and Plasmids—*E. coli* BW313 (Hfr lysA61–62, dut1, ung1, thi1, relA1 spoT1) strain was used for the preparation of single-stranded plasmid DNA prior to mutagenesis (7). *E. coli* ES1301 (lacZ53, mutS201; Tn5, thyA36, rha5, metB1, deoC, IN (rrnD-rrnE)) strain was used for the introduction of mutagenized DNA (8). *E. coli* XL1-Blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,

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**FIG. 1. Schematic diagrams of proposed models for the mechanism of the β-lactamase G238S substitution with cefotaxime in the active site.**

A. Direct hydrogen bond to substrate model in which the serine 238 hydroxyl binds to the oxime group of extended-spectrum antibiotics, such as cefotaxime (5). The steric conflict model in which either B, the B3 β-strand (6) or C, the omega loop (4) is displaced due to steric conflict of the serine hydroxyl with surrounding residues.

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*FIG. 1.* Schematic diagrams of proposed models for the mechanism of the β-lactamase G238S substitution with cefotaxime in the active site. A. Direct hydrogen bond to substrate model in which the serine 238 hydroxyl binds to the oxime group of extended-spectrum antibiotics, such as cefotaxime (5). The steric conflict model in which either B, the B3 β-strand (6) or C, the omega loop (4) is displaced due to steric conflict of the serine hydroxyl with surrounding residues.
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(F′::Tn10[TetR] proAB, lacIq [lacZM15]) strain was used for the preparation of single-stranded DNA and the propagation of plasmid DNA (9). E. coli SB646 (ΔnuA Δspr ΔdegP ΔompT Δprc::kan) is a protease-deficient strain that was used for expression and purification of mutants G238S, G238C, G238T, G238N, and G238V. This strain was a gift from Steve Bass. The plasmid pBG66, described in previous studies (10), was used for mutagenesis. The pBG66 plasmid contains the wild-type bltTEM-1 gene and a cat gene, which encodes for chloramphenicol acetyltransferase. The 4.8-kilobase pair plasmid also contains the ColEl and F origins of DNA replication.

Oligonucleotide-directed Mutagenesis—The G238A, G238C, G238T, G238N, and G238V mutants were prepared in the pBG66 plasmid by Kunkel mutagenesis (7). The G238S mutant was selected directly from the L237–240 random library as a single substitution from an ampicillin antibiotic selection. Oligonucleotide primers used for mutagenesis and DNA sequencing were synthesized by the oligonucleotide synthesis facility at Genentech, Inc., at Stanford University Medical School, and at GenoSys Biotechnologies, Inc. Single-stranded plasmid DNA was prepared for sequencing as described previously (11). DNA sequencing was performed using the dye deoxy chain termination method (12).

Antibiotic Susceptibility—Minimum inhibitory concentrations (MICs) were determined by broth microdilution as described previously (13). The ranges of antibiotic concentrations (2-fold increases) tested were: 128–500 µg/ml for ampicillin and benzylpenicillin (concentration increased 2-fold between 128 and 4096 µg/ml; 3000 and 5000 µg/ml concentrations were added for increased sensitivity), 8–1024 µg/ml for cefaloridine and cefalphathin, 0.03–4 µg/ml for cefotaxime, and 0.12–16 µg/ml for cefoxime.

β-Lactamase Purification—TEM-1 and the residue 238 mutant β-lactamases were purified to >90% homogeneity. The wild-type TEM-1 and G238A enzymes were purified from E. coli XL-1 Blue cells, and the G238S, G238C, G238T, G238N, and G238V enzymes were purified from E. coli SB646 cells. All β-lactamases described were expressed from the pBG66 plasmid that contains the natural β-lactamase promoter. The E. coli SB646 cells were used to increase protein yield for the respective mutants. These strains were grown in culture for at least 8 h to late log phase. The β-lactamase protein was purified as described previously (14). The purity of the enzymes was verified by SDS-polyacrylamide gel electrophoresis.

Enzyme Kinetics—The kinetics of TEM-1 β-lactamase and the residue 238 mutants were determined with ampicillin, benzylpenicillin, cephalexin, cefaloridine, cefalothin, cefotaxime, and ceftazidime. The extinction coefficients used were: ampicillin, 235 nm, ε = 900 M⁻¹ cm⁻¹; benzylpenicillin, 233 nm, ε = 1,140 M⁻¹ cm⁻¹; cephalexin, 260 nm, ε = 10,290 M⁻¹ cm⁻¹; cefaloridine, 262 nm, ε = 7,660 M⁻¹ cm⁻¹; cefotaxime, 264 nm, ε = 7,250 M⁻¹ cm⁻¹ (15), ceftazidime, 260 nm, ε = 8,660 M⁻¹ cm⁻¹ (16). The hydrolysis of the substrates was monitored, and kinetic parameters Vₘₐₓ and Kₘ were determined as described previously (14). The kₐ/Kₘ values for the enzymes where kₐ and Kₘ could not be determined independently were estimated using the equation y = kₐ/Kₘ[S]/[S], where [S] = Kₘ (17). The values reported are based on velocity measurements at 25, 50, and/or 100 µM substrate concentrations.

RESULTS

The “hydrogen bond to substrate” model for the role of residue 238 would predict that, out of the residues tested in this study, only Ser, Thr, and Asn would provide increases in catalytic efficiency of the TEM enzyme for extended-spectrum antibiotics while the “steric conflict” model would predict a direct correlation between an increase in the side chain volume of residue 238 and an increase in catalytic efficiency of the enzyme for extended-spectrum antibiotics. Informational suppression studies done at residue 238 have shown that the G238A and G238T single mutants have increased cefotaxime hydrolytic activity (18), but only the G238S single mutant has been purified to homogeneity and kinetically characterized (19).

To test the models, a series of substitutions with increasing amino acid side chain volume and alternating hydrogen bonding capability (20) were constructed at position 238 (Table I).

For a measure of the biological effect of these substitutions, MICs were determined (Table II) for each mutant with the following substrates: two penicillins (ampicillin and benzylpenicillin), two first generation (nonextended-spectrum) cephalosporins (cephaloridine and cefalothin), and two extended-spectrum cephalosporins (ceftazidime and ceftoxime) (Fig. 2). While the G238S mutant provides a broad spectrum of hydrolytic activity as well as the highest MIC values for the extended-spectrum antibiotics, the G238A, G238T, and G238N mutants have nearly the same hydrolytic activity spectrum, including the extended-spectrum antibiotics, suggesting that these substitutions may be as effective as the Ser substitution in overall antibiotic resistance (Table II). To more clearly determine the effect of each substitution, kinetic analysis was done for each mutant with the six substrates. Because antibiotic resistance, as measured by MICs, has been directly correlated with the catalytic efficiency of the respective antibiotic inactivating enzyme (21), the primary focus of the results will be on the kₐ/Kₘ values. The linear correlation of the catalytic efficiency of the wild-type TEM-1 β-lactamase versus MIC value provided by the enzyme for a given antibiotic is shown in Fig. 3.

Kinetic analysis of the G238A enzyme for penicillins and cephalosporins revealed kₐ/Kₘ values equivalent to the wild-type enzyme for the cephalosporins but at least 2-fold less for the penicillins (Table III). For the extended-spectrum cephalosporins, the overall effect of the G238A substitution is to increase the catalytic efficiency relative to the wild-type enzyme for both ceftazidime (e.g. 2.77 × 10³ versus 3.00 ×10³) and ceftaxime (e.g. 5.51 × 10³ versus 1.77 × 10⁵) (Table III, Fig. 4). These results indicate that a new hydrogen bond interaction is not required to increase hydrolysis of these antibiotics, and therefore provide support for the “steric conflict” model.

The G238S enzyme maintains wild-type levels of kₐ/Kₘ for cephalosporins, while the kₐ/Kₘ values for penicillins exhibits an approximately 10-fold decrease (2.86 × 10⁵ versus 1.12 × 10⁶ for AMP). This can be explained by equivalent reductions in kₐ values for the penicillins and cephalosporins by the G238A enzyme, but reductions in the Kₘ values only for the cephalosporins (Table III). For ceftazidime, as with the other cephalosporins, reduction in the kₐ value is also compensated for by a reduction in Kₘ value, resulting in approximately a 50-fold increase in the kₐ/Kₘ value (e.g. 2.77 × 10³ versus 1.44 × 10⁴) (Table III, Fig. 4). A similar increase in kₐ/Kₘ is seen for ceftazidime. These results are in agreement with published values for the G238S enzyme (19). However, the observation that the Ser residue acts the same way for extended- and nonextended-spectrum cephalosporins suggests that the new interaction introduced is not specific to the oxime group of the extended-spectrum cephalosporins as proposed in the “direct hydrogen bond to substrate” model (Fig. 1A) (5). The results, however, are in agreement with the predictions of the “steric conflict” model (Fig. 1, B and C) (4, 6) because of the increased apparent affinity for all cephalo-

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1. C. Cantu III and T. Palzlilk, unpublished data.

2. The abbreviation used is: MIC, minimum inhibitory concentration.
sporins as indicated by the reduced $K_m$ values (Table III).

The overall effect of the G238C substitution, relative to G238S, is at least a 12-fold decrease in catalytic efficiency for the cephalosporins (e.g. $1.31 \times 10^6$ versus $7.03 \times 10^4$ for cephaloridine and $3.94 \times 10^5$ versus $3.06 \times 10^4$ for cephalothin), but surprisingly little or no change in the efficiency of penicillin hydrolysis (Table III, Fig. 4). For both ceftazidime and cefotaxime hydrolysis, the catalytic efficiency of G238C is at the level of the wild-type enzyme (Fig. 4). These results are not in accordance with the “steric conflict” model. One possibility is that a limitation may have been reached in terms of the volume of the residue 238 side chain such that, for side chains larger than Ala, a hydrogen bond may be necessary for the stabilization of a new active site configuration caused by the steric conflict introduced by the larger side chains. Thus, a hydrogen bonding capability may indeed be required of the residue 238 side chain but for an intramolecular hydrogen bond interaction instead of a direct hydrogen bond to substrate interaction as originally proposed (5).

To further test this model, a valine residue was introduced at position 238. Valine is similar to threonine except for having a methyl group in place of the hydroxyl group. The expectation is that the valine mutant will exhibit a decrease in catalytic efficiency for all antibiotics. In fact, a 10-fold decrease in catalytic efficiency is observed for all G238 mutant enzymes (Table III, Fig. 4).

**Fig. 2.** Structures of β-lactam antibiotics used in this study. These include two penicillins (ampicillin and benzylpenicillin), two first generation (nonextended-spectrum) cephalosporins (cephaloridine and cephalothin), and two extended-spectrum cephalosporins (ceftazidime and cefotaxime).

**Fig. 3.** Linear correlation between the catalytic efficiency of TEM-1 β-lactamase and antibiotic resistance it provides (as measured by MIC values). The x axis represents, on a log scale, the catalytic efficiency, $k_{cat}/K_m$, of the TEM-1 enzyme. The y axis represents the MIC values of E. coli XLI-Blue cells expressing the TEM-1 β-lactamase. Each point represents a single antibiotic. The data, from Tables II and III, are fit to the equation of a line, $y = mx + b$, where $m$ is the slope and $b$ is the y intercept. The dashed line represents the fit with a correlation coefficient of $r = 0.95$. These results clearly support the introduction of a new intramolecular hydrogen bond interaction, as increases in catalytic efficiency are seen for all antibiotics. The results of all the mutants described thus far suggest a model in which the increased side chain volume, due to steric conflict with surrounding residues, leads to an altered active site configuration and that a intramolecular hydrogen bond is needed to stabilize such a configuration as the side chain volume is increased beyond that of alanine.

To further test this model, a valine residue was introduced at position 238. Valine is similar to threonine except for having a methyl group in place of the hydroxyl group. The expectation is that the valine mutant will exhibit a decrease in catalytic efficiency for all antibiotics. In fact, a 10-fold decrease in catalytic efficiency is observed for all G238 mutant enzymes (Table III, Fig. 4).

**Table II**

| MIC | TEM-1 | G238A | G238S | G238C | G238T | G238N | G238V |
|-----|-------|-------|-------|-------|-------|-------|-------|
| AMP | 4096  | 1024  | 1024  | ≤128  | 1024  | 1024  | ≤128  |
| PENG| 4096  | 2048  | 2048  | 256   | 1024  | 2048  | ≤128  |
| CLR | 128   | 128   | 64    | ≤8    | 32    | 128   | ≤8    |
| CF  | 128   | 32    | 64    | 16    | 16    | 32    | ≤8    |
| CTX | 0.06  | 0.12  | 0.5   | 0.12  | 0.12  | 0.12  | 0.06  |
| CAZ | 0.25  | 0.25  | 0.5   | 0.25  | 0.25  | 0.25  | 0.25  |

$^a$ AMP, ampicillin; PENG, benzylpenicillin; CLR, cephaloridine; CF, cephalothin; CTX, cefotaxime; CAZ, ceftazidime.
**Table III**

Kinetic parameters for TEM-1 β-lactamase and its mutants

| MIC $^a$ | TEM-1 $^b$ | G238A | G238S | G238C | G238T | G238N | G238V |
|---------|------------|--------|--------|--------|--------|--------|--------|
| $k_{cat}$ (s$^{-1}$) | | | | | | | |
| AMP     | 1.28 ± 0.02 | 129 ± 6 | 7 ± 1  | 68 ± 18 | 16 ± 1 | 12 ± 5 | 14 ± 1 |
| PENG    | 1.15 ± 0.02 | 91 ± 3  | 3.3 ± 0.4 | 47 ± 4 | 63 ± 14 | 26 ± 3 | 17 ± 2 |
| CLR     | 1.38 ± 0.02 | 149 ± 18 | 6 ± 1  | 21 ± 5  | 80 ± 14 | 25 ± 5 | 21 ± 3 |
| CF      | 1.15 ± 0.02 | 68 ± 25 | 5 ± 1  | 6 ± 1   | 34 ± 4 | 15 ± 2 | 5 ± 1  |
| CTX     | 1.38 ± 0.02 | 41 ± 19 | 18 ± 2 | 4 ± 1   | 16 ± 2 | 6 ± 1  | >2.45 |
| CAZ     | 1.38 ± 0.02 | 41 ± 19 | 18 ± 2 | 4 ± 1   | 16 ± 2 | 6 ± 1  | >2.45 |
| $K_{m}$ (µM) | | | | | | | |
| AMP     | 50 ± 2    | 14 ± 4  | 7 ± 3  | 295 ± 87 | 10 ± 3 | 9 ± 5 | 73 ± 12 |
| PENG    | 75 ± 8    | 10 ± 2  | 12 ± 1 | 93 ± 28  | 47 ± 20 | 9 ± 4 | 63 ± 18 |
| CLR     | 697 ± 12  | 147 ± 39 | 6 ± 2  | 343 ± 19 | 60 ± 29 | 74 ± 24 | 507 ± 75 |
| CF      | 347 ± 14  | 264 ± 34 | 8 ± 5  | 185 ± 47 | 113 ± 13 | 100 ± 22 | 704 ± 187 |
| CTX     | >1500$^c$ | >800$^c$ | >800$^c$ | >800$^c$ | >800$^c$ | >800$^c$ | >800$^c$ |
| CAZ     | >800$^c$  | >800$^c$ | >800$^c$ | >800$^c$ | >800$^c$ | >800$^c$ | >800$^c$ |

$^a$ AMP, ampicillin; PENG, benzylpenicillin; CLR, cephalexin; CF, cephalothin; CTX, cefotaxime; CAZ, ceftazidime.

$^b$ Data from Cantu et al. (13, 14).

$^c$ These values are lower limits. The actual $K_m$ values are outside measurable range. The associated $k_{cat}/K_m$ values are directly estimated under the condition of $[S] << K_m$. The associated $k_{cat}/K_m$ values are derived from the respective $k_{cat}/K_m$ values and the lower limit for the $K_m$ values.

The serine for glycine substitution has been the only one observed at position 238 in natural variants of TEM- and SHV-β-lactamase resistant to extended-spectrum antibiotics (3). The two types of models to explain the development of the G238S substitution in extended-spectrum β-lactamases are: 1) the hydrogen bond to substrate model, where the introduction of a hydrogen bond between the serine hydroxyl and the oxime group of extended-spectrum antibiotics increases antibiotic affinity (Fig. 1A) (5) and 2) the steric conflict model, where the introduction of steric conflict of the serine side chain and surrounding residues would lead to a larger active site cavity and increased affinity for extended-spectrum antibiotics, either by displacement of the B3 β-strand (Fig. 1B) (6) or of the omega loop, which sits at the entrance of the active site (Fig. 1C) (4).

Comparison of inhibitory activities of the extended-spectrum cephalosporins for the residue 238 mutants does not distinguish between the models (Table II). In fact, the Ala, Thr, and Asn mutants have an equivalent spectrum of antibiotic sensitivity as the Ser mutant, which is contrary to the fact that only the Ser substitution appears in extended-spectrum β-lactamases. In nature, selection of the Thr or Asn substitution requires two nucleotide base pair changes in the wild-type Gly$^{238}$ codon while Ser requires only one. This could explain why Ser is preferred over Thr or Asn as the residue 238 substitution in natural variants resistant to extended-spectrum antibiotics.

Although the Ala substitution also requires only one nucleotide base pair change, kinetic analysis of the residue 238 mutants clearly shows that the G238S substitution provides for the best hydrolysis of extended-spectrum cephalosporins and the most versatile hydrolytic spectrum.

While the antibiotic resistance provided by TEM-1 β-lactamase and most of the residue 238 mutants displays a linear correlation ($r = 0.90$–0.99) to the catalytic efficiency of the respective enzyme in agreement with previous studies (21, 22), the correlation breaks down for the G238T mutant ($r = 0.74$) and the G238S mutant ($r = 0.07$). The reason for the breakdown in correlation is not clear but also not unique. Analysis of data from a previous study of the E104K, R164S, and E240K β-lactamase mutants indicate that, individually and in double mutant combinations, they display a linear correlation ($r = 0.92$–1.00) between catalytic efficiency and MIC value, but that correlation breaks down for the triple mutant ($r = 0.58$) (22). Thus, care should be taken before making assumptions of resistance based on catalytic efficiency of an antibiotic inactivating enzyme or vice versa.

Nonetheless, even with the linear correlation breakdown, the G238S mutant still provides the highest values of both catalytic efficiency and MIC for the extended-spectrum cephalosporins. Understanding the factors involved in achieving its high $k_{cat}/K_m$ values for extended-spectrum cephalosporins may provide a better understanding of the mechanism of resistance of extended-spectrum β-lactamases containing the G238S substitution. With regard to the proposed mechanistic models of the G238S substitution, the argument against there being a direct hydrogen bond between the Ser$^{238}$ side chain and the oxime side chain of extended-spectrum cephalosporins is made by the fact that the Ala substitution not only maintains wild-type catalytic efficiency for penicillins and cephalosporins but also increases the catalytic efficiency for extended-spectrum cephalosporins (Fig. 4). Since the introduction of a methyl group results in an increase in $k_{cat}/K_m$ for extended-spectrum drugs,
volume occupancy alone by the residue 238 side chain is an important factor, which lends support to the steric conflict model. Decreased $k_{\text{cat}}/K_m$ values of the Cys and Val mutants for cephalosporins suggest that the Ala side chain volume is an upper limit in providing an increased in catalytic efficiency without the introduction of an additional hydrogen bond interaction, such as with Ser, Thr, or Asn (Fig. 4). The fact that the hydrogen bonding residues provide increases in catalytic efficiency relative to the Cys and Val residues for all the antibiotics tested suggests that the hydrogen bond interaction is not specific to the antibiotic structure but rather causes a change in the active site configuration.

A model to explain these results is that steric conflict of the residue 238 side chain effects the displacement of the omega loop, as proposed by Saves et al. (4), but that an intramolecular hydrogen bond between the residue 238 side chain and an omega loop residue is required to stabilize the omega loop in a new location that allows for significant levels of hydrolysis of extended-spectrum cephalosporins. A possible candidate for the new hydrogen partner of Ser$^{238}$ hydroxyl group is the main chain CO group of Asn$^{170}$ of the omega loop because of its close proximity to residue 238 (23, 24). The G238S mutant has been shown to be destabilized relative to the wild-type TEM-1 enzyme (25), and the lessened stability could be due to the repositioning of the omega loop as a result of a new hydrogen bond between Ser 238 and Asn$^{170}$. Omega loop mutations that provide bacteria increased ceftazidime resistance are thought to do so by destabilizing the loop to allow the larger, bulkier ceftazi-
dime antibiotic greater access to the active site (26, 27). While the Ser substitution would not release omega loop constraints, its larger side chain could be positioned toward Asn$^{170}$ as a result of hydrogen bond formation with this residue and thus displace this region of the loop and perturb the position of the deacetylation residue Glu$^{166}$. This would account for the decreases in $k_{\text{cat}}$ values seen for the penicillins and cephalosporins (Table III). In addition, acylation has been shown to be the rate-limiting step for cefotaxime hydrolysis of both the wild-type TEM-1 and G238S enzymes (4), which means that the $K_m$ value approximates the disassociation value, $K_s$. Comparison of the $K_m$ values of the residue 238 mutants for cefotaxime show that the hydrogen-bonding residues provide the best affinity (Table III). Therefore, the formation of an intramolecular hydrogen bond between the residue 238 side chain and the Asn$^{170}$ main chain CO group may act to tether the omega loop in a new position that allows the extended-spectrum antibiotics greater access to the active site cavity and consequently improved catalytic efficiency by the enzyme.

In summary, the G238S substitution displays an incredible degree of versatility such that it expands the hydrolytic spectrum of the TEM enzyme to include extended-spectrum antibiotics with little cost to its original hydrolytic activity. The manner in which the G238S substitution acts appears to be through an active site cavity expansion by hydrogen bond interaction with the omega loop. As such, distinguishing the mode of action for the change in the active site conformation...
may aid in the design of new \(\beta\)-lactam antibiotics or inhibitors 
or of new combinations of existing drugs.

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