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Reversal of Clavulanate Resistance Conferred by a Ser-244 Mutant of TEM-1 β-Lactamase as a Result of a Second Mutation (Arg to Ser at Position 164) That Enhances Activity against Ceftazidime

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The mutation of Arg-244 to Ser (Arg-244→Ser mutation) in the TEM-1 β-lactamase has been shown to produce resistance to inactivation by clavulanate in the mutant enzyme and resistance to ampicillin plus clavulanate in a strain of *Escherichia coli* producing this enzyme. The Arg-164→Ser mutation in the TEM-1 β-lactamase (TEM-12 enzyme) is known to enhance the activity of the enzyme against ceftazidime, resulting in resistance to the drug in a strain producing the mutant enzyme (D. A. Weber, C. C. Sanders, J. S. Bakken, and J. P. Quinn, J. Infect. Dis. 162:460–465, 1990). The doubly mutated derivative of the TEM-1 enzyme (Ser-164/Ser-244) retains the characteristics of the Ser-164 mutant enzyme, i.e., enhanced activity against ceftazidime and sensitivity to inactivation by clavulanate. It also confers the same phenotype as the Ser-164 mutant enzyme, i.e., resistance to ceftazidime and ampicillin, with reversal of this resistance in the presence of clavulanate. Thus, the Arg-164→Ser mutation in the TEM-1 β-lactamase suppresses the effect of the Arg-244→Ser mutation which, by itself, reduces the sensitivity of the enzyme to inactivation by clavulanate.

The production of β-lactamases is the most common bacterial mechanism of resistance to β-lactam antibiotics. Among clinical isolates of enteric gram-negative bacilli, such as *Escherichia coli*, the most prevalent plasmid-encoded β-lactamase is the prototypical class A (1), or group 2b (4, 5), enzyme, TEM-1 (22). This enzyme is produced constitutively and is principally active against penicillins, such as ampicillin, rather than extended-spectrum cephalosporins, monobactams, or carbapenems (5). However, a single mutation, resulting in the replacement of Arg-164 in TEM-1 by serine to produce the TEM-12 enzyme, is sufficient to enhance enzymatic activity against ceftazidime and aztreonam so that clinically significant resistance to these β-lactams is also conferred (16, 28).

In an attempt to anticipate the possibility that mutation of the TEM-1 β-lactamase could reduce the inactivation of the enzyme by β-lactamase inactivators, we mutagenized a laboratory strain of *E. coli* bearing the TEM-1 β-lactamase with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and grew the mutagenized culture in the presence of inhibitory concentrations of ampicillin and clavulanate (17). In one resistant mutant, the single mutation observed in the structural gene of the enzyme was associated with the replacement of Arg-244 by cysteine. The Cys-244 mutant enzyme and a Ser-244 TEM-1 β-lactamase derivative obtained by site-specific mutagenesis were both resistant to inactivation by clavulanate and conferred resistance to ampicillin and clavulanate (17, 18). Some clinical isolates of *E. coli* resistant to the combination of amoxicillin plus clavulanate have been reported to contain Cys-244 or Ser-244 derivatives of TEM-1 β-lactamase (3, 27). The critical role of Arg-244 in the TEM-1 enzyme in the chemistry of inactivation of clavulanate has been elucidated elsewhere (9).

In reports of extended-spectrum mutants of TEM-1 and related enzymes, it is generally stated that they remain sensitive to inactivation by clavulanate (5, 11), so the option of combining a β-lactamase inactivator with a cephalosporin or aztreonam to preserve antimicrobial activity remains. However, since we know that a single mutation can effectively abolish the sensitivity of TEM-1 β-lactamase to inactivation by clavulanate, we wondered whether an extended-spectrum mutant derivative of TEM-1 β-lactamase, such as TEM-12, could mutate further to resistance to inactivation by clavulanate, thereby conferring resistance to ceftazidime even in the presence of clavulanate. Therefore, we produced the doubly mutated Ser-164/Ser-244 β-lactamase by site-directed mutagenesis and studied the effect of these mutations on the resulting antimicrobial resistance and on the kinetics of the doubly mutated TEM-1 β-lactamase in assays performed with various substrates and clavulanate.

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MATERIALS AND METHODS

β-Lactam drugs. Ampicillin, benzylpenicillin, and cephaloridine were purchased from Sigma. 2-[[[*p*-(Dimethylamino) phenyl]azol-pyridino]cephalosporin (PADAC) was obtained from Calbiochem. Piperacillin, cefotaxime, ceftazidime, and aztreonam were obtained from Lederle, Hoechst, Glaxo, and Squibb, respectively. Clavulanic acid was a gift from Smith-Kline Beecham.

Site-specific mutagenesis of the TEM-1 β-lactamase. The numbering of amino acid residues in the β-lactamase sequence is according to the convention of Ambler et al. (2). The singly mutated derivatives of TEM-1 β-lactamase, the Ser-244 and Ser-164 β-lactamases, and the doubly mutated Ser-164/Ser-144 β-lactamase were prepared from the TEM-1 β-lactamase by site-specific mutagenesis of the gene in pTZ18U (10, 20) by the method of Kunkel (10). The mutated DNA sequences were verified by the dideoxy chain termination method (23). The sequences of all three mutated genes were determined on both strands from nucleotide 424 through nucleotide 858, corre-

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TABLE 1. Susceptibilities of strains to ampicillin and ceftazidime in presence and absence of clavulanate

Drug combination	MIC (μg/ml) for the following strain[b]:				
	MV1190 (no plasmid)	Arg-164 and Arg-244 (TEM-1)	Arg-164 and Ser-244	Ser-164 and Arg-244	Ser-164 and Ser-244
AMP	4	>1,024	>1,024	>1,024	>1,024
AMP + CA (8 μg/ml)	2	16	>1,024	16	16
AMP + CA (16 μg/ml)	1	4	512	4	4
CAZ	0.25	0.25	0.125	128	128
CAZ + CA (8 μg/ml)	0.125	0.125	0.125	0.125	0.125
CAZ + CA (16 μg/ml)	0.125	0.125	0.125	0.125	0.125

[a] AMP, ampicillin; CA, clavulanate; CAZ, ceftazidime.
[b] Strains were *E. coli* MV1190 alone and with pTZ18U containing the gene for the TEM-1 β-lactamase and mutant genes for the Ser-244 enzyme (conferring resistance to inactivation by clavulanate), the Ser-164 enzyme (conferring resistance to ceftazidime), and the Ser-164/Ser-244 double-mutant enzyme.

sponding to the C-terminal half of the enzyme, which includes residues 164 and 244 (162 and 241 in the actual primary sequence of the enzyme). TEM-1 β-lactamase and its mutant derivatives were expressed in *E. coli* MV1190 [Δ(*lac-proAB*) *thi supE* Δ(*srl-recA*)*306*::Tn*10* (*tet*^{r}) (F′:*traD36 proAB lacI*^{q} ZΔM15)] (3a).

β-Lactamase purification. A three-step purification procedure described by Fisher et al. (7) and adapted by Zafaralla et al. (29) was used to purify to homogeneity the following β-lactamases from *E. coli* MV1190 containing the relevant mutations in the TEM-1 β-lactamase gene of pTZ18U: TEM-1, Ser-164 mutant, Ser-244 mutant, and Ser-164/Ser-244 double mutant.

Circular dichroism measurements. Circular dichroic (CD) spectra were recorded on a Jasco model J-600 spectropolarimeter. The protein concentration was 7 μM for all the enzymes, and the buffer was 10 mM sodium phosphate (pH 7.0).

Enzyme kinetics. Kinetic measurements were made with a Perkin-Elmer Lambda 3B or Hewlett-Packard 452 diode-array instrument. The standard spectrophotometric assay of β-lactamase activity was carried out with 50 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl. The initial rates were determined from the first 5 to 10% of the reaction with six or seven substrate concentrations. Rate measurements for each substrate concentration were made in triplicate. The wavelength for each substrate and the change in the extinction coefficient at pH 7.0 were as follows: ampicillin (240 nm), 538 M^{-1} cm^{-1}; cephaloridine (295 nm), 1,000 M^{-1} cm^{-1}; PADAC (466 nm), 9,590 M^{-1} cm^{-1}; piperacillin (240 nm), 780 M^{-1} cm^{-1}; cefotaxime (266 nm), 6,700 M^{-1} cm^{-1}; aztreonam (318 nm), 660 M^{-1} cm^{-1}; and ceftaxidime (260 nm), 10,500 M^{-1} cm^{-1}.

The values for the kinetic parameters (k_{cat} and K_m) were determined from the Lineweaver-Burk plots for the TEM-1 β-lactamase and the Ser-164 mutant, Ser-244 mutant, and Ser-164/Ser-244 double-mutant enzymes. The dissociation constants (K_i) for clavulanate with the TEM-1 and mutant enzymes were calculated by the method of Dixon (6a) according to the protocol of Imtiaz et al. (9). Two concentrations of the substrate ampicillin, 400 and 500 μM, were used for the determination of K_i for clavulanate with the wild-type and Ser-244 mutant β-lactamases. Since the k_{cat} for ampicillin with the Ser-164 and Ser-164/Ser-244 enzymes was found to be significantly lower than that with the TEM-1 and Ser-244 enzymes (see Table 2), it was inconvenient to use penicillin as a substrate for monitoring hydrolysis by these enzymes. Therefore, for the Ser-164 and Ser-164/Ser-244 enzymes, PADAC was used instead of ampicillin, at concentrations of 100 and 200 μM. Because of the large change in the extinction coefficient, the hydrolysis of PADAC was monitored with ease. A series of assay mixtures containing both the substrate and various concentrations of clavulanate (0.5 to 4.0 μM for the TEM-1 β-lactamase, 2.5 to 50 μM for the Ser-244 enzyme, and 0.02 to 0.25 μM for the Ser-164 and Ser-164/Ser-244 enzymes) were prepared with 100 mM sodium phosphate buffer (pH 7.0). The enzyme was added to the reaction mixture, and its activity was determined. Rates were calculated from the initial linear portion corresponding to approximately the first 5% of substrate turnover.

The partition ratios for clavulanate with the TEM-1 and mutant enzymes were determined by the titration method (24). Several buffered mixtures containing various molar ratios of inactivator to enzyme ranging from 1 to 200 for the TEM-1 β-lactamase, 1 to 8,000 for the Ser-244 enzyme, and 1 to 100 for the Ser-164 and Ser-164/Ser-244 enzymes were incubated at 4°C in 100 mM sodium phosphate buffer (pH 7.0). After approximately 20 h, the remaining activity of the TEM-1 and Ser-244 enzymes was assayed by monitoring the hydrolysis of 2 mM benzylpenicillin at 240 nm upon the addition of an aliquot of an incubated mixture of enzyme and clavulanate. For the Ser-164 and Ser-164/Ser-244 enzymes, the same procedure was followed, except that, for reasons mentioned above, PADAC (1 mM) was used instead of benzylpenicillin for monitoring substrate hydrolysis. The extent of nonspecific inactivation of the enzymes was taken into account by means of a control experiment in which enzyme activity was measured under identical conditions but in the absence of an inactivator.

Determination of MICs. The MICs of ampicillin and ceftazidime with and without clavulanate for *E. coli* MV1190 and transformants containing pTZ18U with a wild-type or mutant β-lactamase gene were determined by the broth microdilution method with inocula of 5×10^5 CFU/ml in Mueller-Hinton broth (12). The MIC was defined as the lowest concentration of drug that prevented the appearance of turbidity after 24 h of incubation at 37°C.

RESULTS

Effects of mutations in the TEM-1 β-lactamase on resistance conferred by the enzyme. Table 1 compares the MICs of ampicillin and ceftazidime in the presence and absence of clavulanate for *E. coli* MV1190 and transformants containing pTZ18U with the wild-type gene for the TEM-1 β-lactamase and mutant genes for the Ser-244 enzyme, the Ser-164 enzyme, and the Ser-164/Ser-244 double-mutant enzyme. MV1190 was susceptible to ampicillin and even more susceptible to the extended-spectrum cephalosporin ceftazidime. As expected, the TEM-1 enzyme conferred high-level resistance to ampicillin, and the presence of clavulanate at 8 and 16 μg/ml reduced the MIC of ampicillin drastically. The presence of the TEM-1

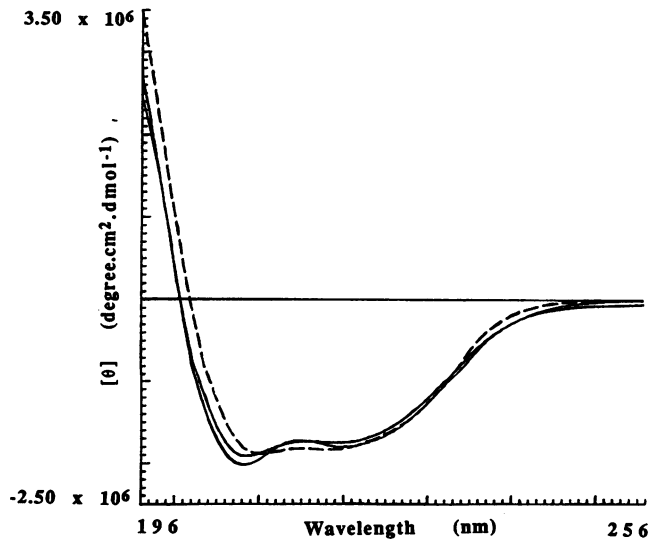

FIG. 1. CD spectra of the wild-type TEM-1 β-lactamase (broken line) and of the Ser-164 and Ser-164/Ser-244 mutant derivatives (solid lines). θ, molar ellipticity.

β-lactamase had no effect on the MIC of the poor substrate ceftazidime. As shown previously (18, 19), the replacement of Arg-244 by Ser (Arg-244→Ser) drastically reduced the effect of clavulanate, so that a strain bearing the mutant enzyme was highly resistant to the combination of ampicillin plus clavulanate. As reported previously (28), the mutant enzyme with the Arg-164→Ser mutation conferred high-level resistance to ceftazidime that was reversed by clavulanate. The double-mutant TEM-1 β-lactamase, Ser-164/Ser-244, conferred resistance to both ampicillin and ceftazidime, but this resistance to both β-lactams was reversed by clavulanate, despite the presence of the Arg-244→Ser mutation. Thus, the resistance phenotype conferred by the Ser-164/Ser-244 double-mutant enzyme was the same as that conferred by the Ser-164 enzyme.

Circular dichroism. The CD spectra of the purified TEM-1 β-lactamase and the Ser-164 mutant and Ser-164/Ser-244 double-mutant enzymes are shown in Fig. 1. We had shown earlier that the CD spectrum of the Ser-244 mutant enzyme is superimposable on that of the TEM-1 enzyme at pH 4.0 to 9.0 (29). Therefore, it would appear that no change in the overall collective secondary structural elements of the protein resulted from mutagenesis at position 244. On the other hand, the results shown in Fig. 1 indicated that the secondary structural elements of the TEM-1 β-lactamase were affected by the Arg-164→Ser mutation. Similarly, the CD spectrum of the Ser-164/Ser-244 double-mutant enzyme was found to be different from that of the TEM-1 β-lactamase but similar to that of the Ser-164 mutant enzyme.

Kinetics of hydrolysis. The kinetic parameters for the hydrolysis of various β-lactam substrates were evaluated for the TEM-1 β-lactamase and the Ser-244 mutant, Ser-164 mutant, and Ser-164/Ser-244 double-mutant β-lactamases (Table 2). The k_{cat} values measured for the Ser-164 mutant enzyme with the penicillin substrates were significantly lower than those measured for the wild-type TEM-1 enzyme; reductions of 88- and 53-fold were noted for ampicillin and piperacillin, respectively. The k_{cat} values were also reduced by 25- and 40-fold, respectively, for PADAC and cephaloridine. There was an accompanying decrease in K_m values, although it was less pronounced than the decrease in the k_{cat} values. A decrease of no more than fourfold was observed in the K_m values for all of the above-mentioned substrates. Consequently, the resulting decrease in k_{cat}/K_m values for the Ser-164 mutant enzyme, in comparison with those for the wild-type β-lactamase, was in the range of 12- to 17-fold for piperacillin, PADAC, and cephaloridine. However, a greater reduction, that of 67-fold, was observed in the k_{cat}/K_m value for ampicillin. In contrast to those for the penicillin substrates and the cephalosporin substrates PADAC and cephaloridine, the k_{cat}/K_m values for cefotaxime and aztreonam were found to be two- and threefold higher, respectively, for the Ser-164 mutant enzyme than for the TEM-1 β-lactamase. Furthermore, there was a dramatic increase of more than 2 orders of magnitude in the k_{cat}/K_m value for ceftazidime.

TABLE 2. Kinetic parameters[a] for the hydrolysis of substrates by the wild-type TEM-1 enzyme and the Ser-164, Ser-244, and Ser-164/Ser-244 mutant enzymes

β-Lactam substrate	TEM β-lactamase	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\ s^{-1}$)
Ampicillin	Wild type	1,359	49	2.8×10^7
	Ser-244	1,453	352	4.1×10^6
	Ser-164	15.4	36.7	4.2×10^5
	Ser-164/Ser-244	27.5	41.3	6.7×10^5
Piperacillin	Wild type	592.9	70.6	8.4×10^6
	Ser-244	767.2	450	1.7×10^6
	Ser-164	11.1	22.3	5.0×10^5
	Ser-164/Ser-244	11.8	17	6.9×10^5
PADAC	Wild type	400	98	4.1×10^6
	Ser-244	64.8	684	9.5×10^4
	Ser-164	16	53.8	3.0×10^5
	Ser-164/Ser-244	32.9	76.6	4.3×10^5
Cephaloridine	Wild type	935	785	1.2×10^6
	Ser-244	112.4	3,700	3.0×10^4
	Ser-164	22.4	239	9.4×10^4
	Ser-164/Ser-244	29	281	1.0×10^5
Cefotaxime	Wild type	0.13	220	5.9×10^2
	Ser-244	ND[b]	ND	ND
	Ser-164	0.2	174	1.1×10^3
	Ser-164/Ser-244	0.18	228	7.9×10^2
Ceftazidime	Wild type	ND	ND	$<2.5 \times 10^1$
	Ser-244	ND	ND	$<1.0 \times 10^1$
	Ser-164	1.15	257	4.5×10^3
	Ser-164/Ser-244	2.6	327	8.0×10^3
Aztreonam	Wild type	0.17	204	8.3×10^2
	Ser-244	ND	ND	ND
	Ser-164	1.5	635	2.4×10^3
	Ser-164/Ser-244	1.69	507	3.3×10^3

[a] The standard deviation for analysis was ±5%.
[b] ND, not detected.

The kinetic comparison of substrate hydrolysis between the wild-type TEM-1 and Ser-244 mutant β-lactamases revealed a drop in the catalytic efficiency (k_{cat}/K_m) of the enzyme by 5- to 7-fold for the two penicillins tested and approximately 40-fold for the cephalosporins PADAC and cephaloridine. Ceftazidime, aztreonam, and cefotaxime were found to be extremely poor substrates for the Ser-244 mutant enzyme. Therefore, the k_{cat} and K_m values for these substrates could not be evaluated. In the case of ceftazidime, however, an estimation on the upper limit for the ratio k_{cat}/K_m was made from the first-order

TABLE 3. Inhibitor constants for clavulanate and efficiency of inactivation by clavulanate for the TEM-1 β-lactamase and the Ser-244, Ser-164, and Ser-164/Ser-244 mutant β-lactamases

TEM β-lactamase	K_i (μM)[a]	$[I]_0/[E]_0$[b]
TEM-1	0.4[c]	150
Ser-244	33[c]	7,500
Ser-164	0.15	20
Ser-164/Ser-244	0.25	50

[a] The standard deviations for these determinations were ±10%.
[b] Ratio at which 5% of the initial activity remained.
[c] Reported by Imtiaz et al. (9).

rate of hydrolysis of the substrate under conditions of $[S]_0 < K_m$.

The k_{cat}/K_m values obtained for the double-mutant enzyme were, in comparison with those obtained for the wild-type enzyme, lower for all of the substrates, except for the ones with an aminothiazole oxime side chain in their structures. Furthermore, the kinetic parameters for substrate hydrolysis by the double-mutant enzyme (Ser-164/Ser-244) were found to be very similar to the respective kinetic parameters obtained for the Ser-164 mutant enzyme. Indeed, for all of the substrates tested, no more than a twofold difference was noted between the k_{cat}/K_m values obtained for the Ser-164 and Ser-164/Ser-244 mutant β-lactamases. Thus, the effect of a mutation at position 164 seems to be dominant for the hydrolytic reaction over the effect of a mutation at residue 244.

Kinetics of enzyme inactivation by clavulanate. Earlier work in our laboratories (9) had shown that the Arg-244→Ser mutation results in a decreased affinity of the enzyme for clavulanate. The K_i values determined for clavulanate with the TEM-1 β-lactamase and the Ser-244, Ser-164, and Ser-164/Ser-244 mutant enzymes are shown in Table 3. The K_i value for clavulanate was found to be 82-fold higher with the Ser-244 mutant enzyme than with the wild-type enzyme. In contrast, the K_i values for clavulanate were both found to be approximately two- to threefold lower with the Ser-164 and Ser-164/Ser-244 mutant enzymes than with the wild-type enzyme (Table 3).

The partition ratio determinations for clavulanate with the wild-type and Ser-244 mutant enzymes had been reported earlier (9). A linear plot of $[I]_0/[E]_0$ versus $100\{([E]_0 - [E_{inact}]_\infty)/[E]_0\}$ was obtained for the TEM-1 enzyme, allowing for the determination of a partition ratio of 160. For the Ser-244 mutant enzyme, however, the plot of $[I]_0/[E]_0$ versus $100\{([E]_0 - [E]_{inact}]_\infty)/[E]_0\}$ resulted in a curve that was linear only initially and subsequently deviated from linearity to approach a hyperbola. Although extrapolation of the initial linear portion of the plot yielded a partition ratio of 190, in reality complete inactivation did not result, even with an $[I]_0/[E]_0$ ratio of 8,000. Because of the nonlinear nature of the plots of $[I]_0/[E]_0$ versus $100\{([E]_0 - [E_{inact}]_\infty)/[E]_0\}$ for all three mutant enzymes in this study, accurate partition ratios could not be determined in these cases. Furthermore, because of the excessively high concentrations of compound required, experiments were not carried out for a range of $[I]_0/[E]_0$ ratios of >8,000 for the Ser-244 enzyme; such experiments would be required to determine the ratio at which complete inactivation of the enzyme would result. Nevertheless, a valid comparison of the ability of clavulanate to inactivate the wild-type and mutant β-lactamases can still be made by comparing the $[I]_0/[E]_0$ ratios at which only a certain low percentage of enzyme activity remains. Table 3 lists the $[I]_0/[E]_0$ ratios at which only 5% of the enzyme activity remained after incubation with clavulanate for 20 h.

The Ser-244 mutant β-lactamase is known to be resistant to inactivation by clavulanate (3, 6, 9, 19, 20). This is evident from the high $[I]_0/[E]_0$ ratio at which only 5% of the enzyme activity remained (i.e., 7,500 for the Ser-244 enzyme); the corresponding ratio for the wild-type TEM-1 β-lactamase was only 150 (Table 3). In contrast, the Ser-164 mutant β-lactamase appeared somewhat more sensitive to inactivation by clavulanate than the wild-type TEM-1 β-lactamase. The corresponding ratio for the Ser-164/Ser-244 double-mutant enzyme was only 50, indicating that it was also somewhat more sensitive to inactivation by clavulanate than the wild-type enzyme.

DISCUSSION

Arginine 164 is a conserved residue in all class A β-lactamases (2). From the crystal structure of class A β-lactamases (8, 13, 15, 21, 26), it is apparent that the side chain of Arg-164 is involved in a salt bridge with the side chain of Asp-179, another conserved residue in class A β-lactamases, and eight structurally conserved water molecules are associated with these residues. Since both Arg-164 and Asp-179 are conserved residues in class A β-lactamases, the salt bridge between these two residues must serve an essential function by stabilizing the Ω loop that they form. We have shown, in confirmation of the report by Sowek et al. (25), that the Arg-164→Ser mutation, which would be expected to disrupt the salt bridge, lowers the catalytic efficiency of the mutant enzyme for all of the substrates tested, except for those with an aminothiazole oxime side chain. In fact, the k_{cat}/K_m value for ceftazidime was almost 200-fold higher for the Ser-164 mutant enzyme than for the wild-type TEM-1 β-lactamase and was even higher for the Ser-164/Ser-244 double-mutant enzyme. This enhanced activity for ceftazidime of both of these mutant enzymes which contain Ser-164 resulted in clinically significant high levels of resistance to ceftazidime in strains bearing either of them. As in the initial report of the TEM-12 β-lactamase (28), the resistance to ceftazidime conferred by this enzyme (Ser-164) was reversed by clavulanate.

Arginine 244 is also a highly conserved residue in class A β-lactamases. No structural role for this residue is apparent from the crystal structure of the *Bacillus licheniformis* enzyme, so a mechanistic function has been considered likely to account for its high conservation (29). It has been shown that the guanidinium moiety of Arg-244 is involved in a long hydrogen bonding interaction with the carboxylate of penicillin and cephalosporin substrates (29). Recent crystallographic studies of a deacylation-defective mutant (Asn-166) TEM-1 β-lactamase (26) have also revealed the hydrogen bonding interaction between Arg-244 and the acyl-enzyme intermediate for penicillin G.

Mutagenesis of Arg-244 in the TEM-1 β-lactamase to serine or cysteine results in resistance to inactivation by clavulanate (3, 6, 9, 18, 19). To understand the crucial role of this highly conserved residue in the chemistry of inactivation of clavulanate, we carried out a detailed structure-based analysis of clavulanate chemistry by using the high-resolution crystal structure of the *B. licheniformis* 749/C β-lactamase (13) for the modelling studies (9). On the basis of the findings from those studies, we proposed a nonconcerted process for the clavulanate inactivation chemistry. The side-chain guanidinium moiety of Arg-244, along with the main-chain carbonyl moiety of Val-216, anchors a structurally conserved water molecule, Wat-673. The modelling studies revealed that this water molecule may donate a critical proton in the clavulanate inactiva-

tion chemistry. A mutant β-lactamase with serine or cysteine at position 244 would no longer be able to retain Wat-673 with a high affinity. The absence of this essential water molecule from its ideal position in the mutant enzymes resulted in a significant compromise in the ability of clavulanate to inactivate the enzymes. This effect was expressed in vivo by a marked diminution in the reversal of resistance to ampicillin brought about by the addition of clavulanate to a culture of an *E. coli* strain bearing the Ser-244 mutant β-lactamase, in comparison with the susceptibility of an isogenic strain bearing the wild-type TEM-1 β-lactamase (Table 1). Further in vivo kinetic characterization of this mutant enzyme showed not only a decrease in the affinity for clavulanate but also serious retardation in the inactivation chemistry (Table 3).

Unexpectedly, our results in Table 1 showed that the Ser-164/Ser-244 double-mutant TEM-1 β-lactamase failed to confer resistance to ampicillin or ceftazidime plus clavulanate, despite the presence of Ser-244. The resistance to ceftazidime conferred by the Ser-164 mutant enzyme was preserved in the double-mutant enzyme. Thus, the Arg-164→Ser mutation is an intracistronic suppressor of the clavulanate resistance phenotype produced by the Arg-244→Ser mutation.

The reversal of ampicillin resistance by clavulanate in a strain bearing the Ser-164/Ser-244 double-mutant enzyme, despite the presence of Ser-244, is explained by the observation that the double-mutant enzyme remained sensitive to inactivation by clavulanate, like the wild-type and Ser-164 mutant enzymes (Table 3). Since mutation to serine at residue 244 would alter the position of Wat-673, which provides an essential proton in the clavulanate inactivation chemistry, it is not obvious at present why the double-mutant enzyme was sensitive to clavulanate. However, it is evident from the CD spectra of the mutant proteins that, whereas no change in the overall collective secondary structural elements of the protein was noted for the Ser-244 mutant enzyme, the Ser-164 mutant enzyme apparently had undergone a change. Similarly, the CD spectrum of the double-mutant enzyme was found to be different from that of the wild-type β-lactamase but similar to that of the Ser-164 mutant enzyme. It is therefore possible that the disruption of the Ω loop, as a result of the mutation at position 164 in the Ser-164 and Ser-164/Ser-244 mutant β-lactamases, causes an alteration in the topology of the active sites of these enzymes so that they may bind to clavulanate in a somewhat different manner. Although the nature of the topological alteration is unknown, another conserved residue or an active-site water molecule may be brought close to the site of the requisite protonation of clavulanate and may thereby serve to donate the essential proton for inactivation by clavulanate.

The effect of the Arg-164→Ser mutation that enhances catalytic activity for ceftazidime and confers resistance to it seems to be dominant over that of the Arg-244→Ser mutation with regard to the hydrolytic properties of the double-mutant TEM-1 β-lactamase and its inactivation by clavulanate. As a practical consequence, it thus appears that reversal by clavulanate of the resistance to ceftazidime that is conferred by the Ser-164 mutant enzyme (i.e., TEM-12) and perhaps by other TEM derivatives that contain Ser-164 is not eliminated by a second mutation (Arg-244→Ser) which would by itself nullify the utility of clavulanate against strains bearing the TEM-1 enzyme.

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