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Replacement of Lysine 234 Affects Transition State Stabilization in the Active Site of β-Lactamase TEM1*

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Lysine 234 is a residue highly conserved in all β-lactamases, except in the carbenicillin-hydrolyzing enzymes, in which it is replaced by an arginine. Informational suppression has been used to create amino acid substitutions at this position in the broad spectrum *Escherichia coli* β-lactamase TEM-1, in order to elucidate the role of this residue which lies on the wall at the closed end of the active site cavity. The mutants K234R and K234T were constructed and their kinetic constants measured.

Replacement of lysine 234 by arginine yields an enzyme with similar activity toward cephalosporins and most penicillins, except toward the carboxypenicillins for which the presence of the guanidine group enhances the transition state binding . The removal of the basic group in the mutant K234T yields a protein variant which retains a low activity toward penicillins, but losts drastically its ability to hydrolyze cephalosporins. Moreover, these two mutations largely decreased the affinity of the enzyme for penicillins (10-fold for K234R and 50-fold for K234T). This can be correlated with the disruption of the predicted electrostatic binding between the C3 carboxylic group of penicillins and the amine function of the lysine.

Therefore, lysine 234 in the *E. coli* β-lactamase TEM-1 is involved both in the initial recognition of the substrate and in transition state stabilization.

The most common mechanism of resistance to β-lactams is the production of β-lactamases, in both $Gram^+$ and $Gram^-$ bacteria. Understanding the molecular details of the interactions between the β-lactamases and their substrates, the β-lactam antibiotics, could help draw a precise picture of the structure-function relationships within the active site. This should prove an invaluable tool for the design of new antibiotics.

On the basis of comparison of the primary structures of a few β-lactamases, the different enzymes have been grouped into four classes (1), A, B, C, and D, of which the β-lactamases of the class A are the most commonly encountered in clinical isolates. The sequence alignments have defined seven highly conserved "boxes" (2). The crystallographic data of the class A β-lactamases from Gram-positive bacteria *Staphylococcus aureus* PC1 (3), *Streptomyces albus* G (4), and more recently the high resolution structure of *Bacillus licheniformis* 749/C β-lactamase at 2.0 Å (5) suggest strongly that the sequence homologies of class A β-lactamases can be related to high tertiary structural and functional similarities.

The β-lactamases involve an active site serine residue, Ser-70 (using Ambler's numbering) and the hydrolysis of the β-lactam ring is catalyzed via an acyl-enzyme intermediate (6). It has been suggested that both lysine 73 and glutamic acid 166 play a part in the mechanism. Oligonucleotide-directed mutagenesis performed at these positions resulted in protein variants with drastic loss of activity (7, 8).[1] The role of the Ser-Asp-Asn region has just been investigated (9). On the β3 strand delimiting the active site, residues were also identified as playing a role in substrate binding or in substrate specificity (10–12). Lysine 234 is located, on this β3 strand, in the highly conserved triad Lys-Ser-Gly or Lys-Thr-Gly (box VII) both in β-lactamases and in most penicillin-binding proteins. The crystallographic data (3, 5) have shown that this highly conserved amino acid is localized in the wall at the closed end of the active site depression, and its contribution to the initial binding of the antibiotic in the catalytic cavity was postulated. Recently, site-directed mutagenesis was used by Ellerby *et al.* (13) to substitute the lysine 234 with glutamic acid or alanine in the β-lactamase from *B. licheniformis*, demonstrating its importance in catalysis. However, in most known carbenicillin-hydrolyzing enzyme sequences (14), this lysine is replaced by an arginine. All the models that are proposed on the interaction of the class A enzymes with their substrate are built from β-lactamases from $Gram^+$ organisms and penicillin. It remains to be seen whether this can be fully applied to enzymes from $Gram^-$ bacteria, which usually exhibit a broader substrate spectrum including cephalosporins.

To investigate further the role of lysine 234 in the binding and catalysis of β-lactam hydrolysis by one such broad spectrum β-lactamase, the enzyme TEM-1, we used informational suppression. This is a powerful tool to investigate the effects of various amino acid substitutions at a given position in a protein. With the new extended set of amber suppressor strains (15–17), this method has been used recently to create variants of the *lac* repressor (18), to investigate the thermostability of α-amylase (19) or to study new substrate specificity in TEM-1 β-lactamase (11). We likewise generated multiple amino acid substitutions of this lysine 234 in the β-lactamase TEM-1 and investigated the phenotype of the variants producing strains. This informational suppression experiment suggested to us the substitution of lysine 234 by a threonine, in addition to the more conservative substitution by an arginine.

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[1] M. Delaire, F. Lenfant, R. Labia, and J. M. Masson, unpublished results.

MATERIALS AND METHODS

E. coli Strains and Plasmids—Bacterial strains used were *E. coli* XaC-1: F′ $lacI_{373}$ $lacZ_{u118am}$ $proB^+$/(*lac-pro*)$_{\times 111}$ *nalA rif* $argE_{am}$ *ara* or its derivatives expressing a $tRNA_{CUA}$ suppressor gene. They have been described previously (11). Suppressors employed here are Sul, Su2–89, Su3, Su6, and GlyU inserting serine, glutamine, tyrosine, leucine, and glycine, respectively, and the synthetic suppressor genes Ala-$tRNA_{CUA}$, Cys-$tRNA_{CUA}$, GluA-$tRNA_{CUA}$, His-$tRNA_{CUA}$, Lys-$tRNA_{CUA}$, Pro-$tRNA_{CUA}$, Phe-$tRNA_{CUA}$, Thr-$tRNA_{CUA}$ (15–17). The Arg-$tRNA_{CUA}$ is made from a modified phenylalanine inserting gene (20). To provide compatibility with any plasmid having a ColE1 replicative origin, the *Hin*dIII-*Hin*cII fragments from pGFIB1 (21), carrying the synthetic suppressors have been recombined with the *Hin*dIII-*Hin*cII fragment of pCTB2 (11), a derivative from pACYC184. These nine plasmids pCTB2-Sup were introduced into the *E. coli* XaC-1 strain.

On the plasmid pCT1 already described (11), the *bla* TEM-1 gene is transcribed from the pUC *bla* promoter. To obtain a 10-fold increase in the expression of the β-lactamase TEM-1 in *E. coli*, the stronger promoter Pa+Pb (22) was cloned in front of the *bla* gene by substituting the *Ssp*I-*Pst*I fragment from plasmid pCT1 by the same fragment from plasmid pT28C2 encoding the mutant *bla* gene T284A (23). The resulting plasmid was called pCT3.

Media, Antibiotics, and Enzymes—Cells were grown in LB medium supplemented with the appropriate antibiotics: 100 μg/ml ampicillin (Sigma), 30 μg/ml chloramphenicol (Serva), and 12 μg/ml tetracycline (Boehringer Mannheim). [^{35}S]dCTP was provided by Amersham Corp. All enzymes for genetic engineering were obtained from Pharmacia LKB Biotechnology Inc.

Antibiotic powders were a gift from their respective manufacturers: benzylpenicillin (Rhone-Poulenc), amoxicillin, ticarcillin, carbenicillin, clavulanic acid (Beecham), piperacillin (Lelerle), cephalothin (Lilly), cephaloridine (Glaxo), cefoperazone (Pfizer), cefotaxime (Roussel-Uclaf). Phenoxymethylpenicillin was purchased from Sigma.

Bacterial strains producing the different protein variants were assayed for growth in the presence of various β-lactam antibiotics. Assays were first done using the disc diffusion technique on LB agar plates. Paper discs (Diagnostics Pasteur) impregnated with the indicated amount of antibiotic (25 μg of amoxicillin, 20 μg of amoxicillin + 10 μg of clavulanic acid, 75 μg of ticarcillin, 75 μg of ticarcillin + 10 μg of clavulanic acid, 30 μg of cephalothin, 30 μg of cefoperazone, and 30 μg of cefotaxime) were placed on a lawn of growing cells, and the area of inhibited growth around the discs was measured.

Recombinant DNA Techniques—Cloning techniques were based on Maniatis *et al.* (24). DNA sequencing was carried out using the Pharmacia T7 polymerase sequencing kit. The preparation of competent *E. coli* cells and subsequent transformation with plasmid DNA were carried out according to the protocol of Hanahan (25).

Oligonucleotides and Site-directed Mutagenesis—Synthetic oligonucleotides were made as trityl derivatives on an Applied Biosystem 380B DNA synthesizer using phospharamidite chemistry and purified on Nensorb Prep Columns as specified by the manufacturer (I. E. Du Pont de Nemours & Co.). The following oligonucleotides were used:

K234am 5′ ACCGGCTCCACT**CTA**ATCAGCAAT 3′
K234R 5′ ACCGGCTCCAGA**CCG**ATCAGCAAT 3′
K234T 5′ ACCGGCTCCAGA**CGT**ATCAGCAAT 3′

The codon downstream from the amber codon was changed from TCT to AGT to ensure a better suppression context without changing the protein sequence.

Oligonucleotides were phosphorylated as previously described (15). The single-stranded pCT1 DNA was isolated after infection by the helper phage M13K07 (Pharmacia). The site-directed mutagenesis was then accomplished using Eckstein's method (26). The mutant colonies were selected for their loss of resistance to ampicillin. Missense revertants were obtained by the same manner, starting from the amber mutated genes. The mutations were confirmed by sequencing the entire gene to check that no undesired mutation had been introduced. This sequencing was performed using single-stranded pCT1 DNA and six internal oligonucleotides as primers.

β-Lactamase Expression and Purification—The expression vector is plasmid pCT3. Bacterial cells were grown at 30 °C in LB medium supplemented with 0.02% glycerol, 20 mM $MgSO_4$, 100 μg/ml ampicillin, and 12 μg/ml tetracycline. β-Lactamase was extracted by osmotic shock, as previously described (27).

The β-lactamase was then purified from 1 liter of culture by preparative electrofocusing using the Multiphor II system (LKB) on a 4–6.5 pH gradient. After elution with 10 ml of 50 mM sodium phosphate buffer, pH 7, gel filtration on a Sephacryl HR100 (Pharmacia) column was further performed to reach a high level of purity. The fractions containing the enzyme were detected using the nitrocefin assay (28) in 50 mM buffer, at 37 °C (ϵ = 20,500 $M^{-1} \cdot cm^{-1}$). Protein concentration was determined by absorbance measurements at 280 nm, with ϵ = 18,200 $M^{-1} \cdot cm^{-1}$ obtained for the wild type enzyme. The homogeneity of the protein can be estimated to be >95% on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric point (pI) was determined on analytical isoelectric focusing revealed by the iodine procedure, in agar gel with benzylpenicillin as substrate (29).

Determination of the Kinetic Parameters of the Mutant Enzymes—The kinetic constants k_{cat} and K_m for various substrates were determined by computerized microacidometry (30). When the K_m value was too high, only k_{cat}/K_m could be obtained by using a nonlinear least square fit regression for one exponential curve at $[S] \ll K_m$.

The affinity of the enzyme for the inhibitor, which is expressed as the inhibition constant K_i, was measured using competition procedures with benzylpenicillin. It is determined from the extrapolated rate, at the time when the inhibitor is added. Inhibition by clavulanic acid was tested by incubation with the enzyme for 10 min at 37 °C before measuring the remaining enzymatic activity. I_{50} value was defined as the concentration of inhibitor causing 50% inhibition of benzylpenicillin hydrolysis by the enzyme. As the measures were done with highly purified proteins, the value of I_{50} was used to calculate the stoichiometry of the reaction in order to access to the turnover number. The inactivation constant k_{inac} was also deduced from incubation of the inhibitor with the enzyme for various times. A large excess of benzylpenicillin was thus added and the remaining rate was monitored. The measure of the half-life time of inactivation then allowed us to calculate the k_{inac} value ($k_{inac} = ln2/t_{1/2}$ when $[S] \gg K_m$).

One unit of β-lactamase activity is defined as the amount of enzyme hydrolyzing 1 μmol of substrate/min at pH 7 and 37 °C.

RESULTS

Multiple Amino Acid Substitutions of Lysine 234 and Expression in E. coli

To perform the multiple amino acid substitutions of lysine 234, the amber codon "TAG" was first introduced at the corresponding codon "AAA" into the *bla* gene TEM-1 by oligonucleotide-directed mutagenesis. After the insertion of the highly efficient promoter Pa+Pb of T284A gene in front of the mutated *bla* gene, the plasmid pCT3 was introduced in the 14 available strains harboring an amber suppressor gene. Fourteen protein variants at the position 234 were thus generated. The activity of each protein variant was then characterized phenotypically by antibiotic disc assays with respect to five different antibiotics: two penicillins, amoxicillin and ticarcillin, alone or in combination with clavulanic acid; a first generation cephalosporin, cephalothin; two third generation cephalosporins, cefoperazone and cefotaxime (Fig. 1).

Table I reports the values of the inhibition diameters as determined by antibiograms. It can first be observed that the strain expressing the mutated gene in the absence of suppressor presented no β-lactamase activity. Immunoblots revealed no detectable amount of the enzyme (data not shown), indicating that the truncated unfunctional protein was unstable or could not be exported to the periplasmic space. In other respects, the strain overexpressing the amber mutated gene was found to grow very slowly. The substitution of lysine 234 by the different amino acids yielded a large number of mutants demonstrating a very low but detectable activity. The strains producing the protein variants K234G, K234E, K234C, K234Y, and K234S exhibited some activity toward amoxicillin but far from a true resistance phenotype. Surprisingly, these β-lactamase mutants correspond to substitutions by amino acids with polar chains. Suppression efficiency can vary widely depending both on the type of suppressor used and the

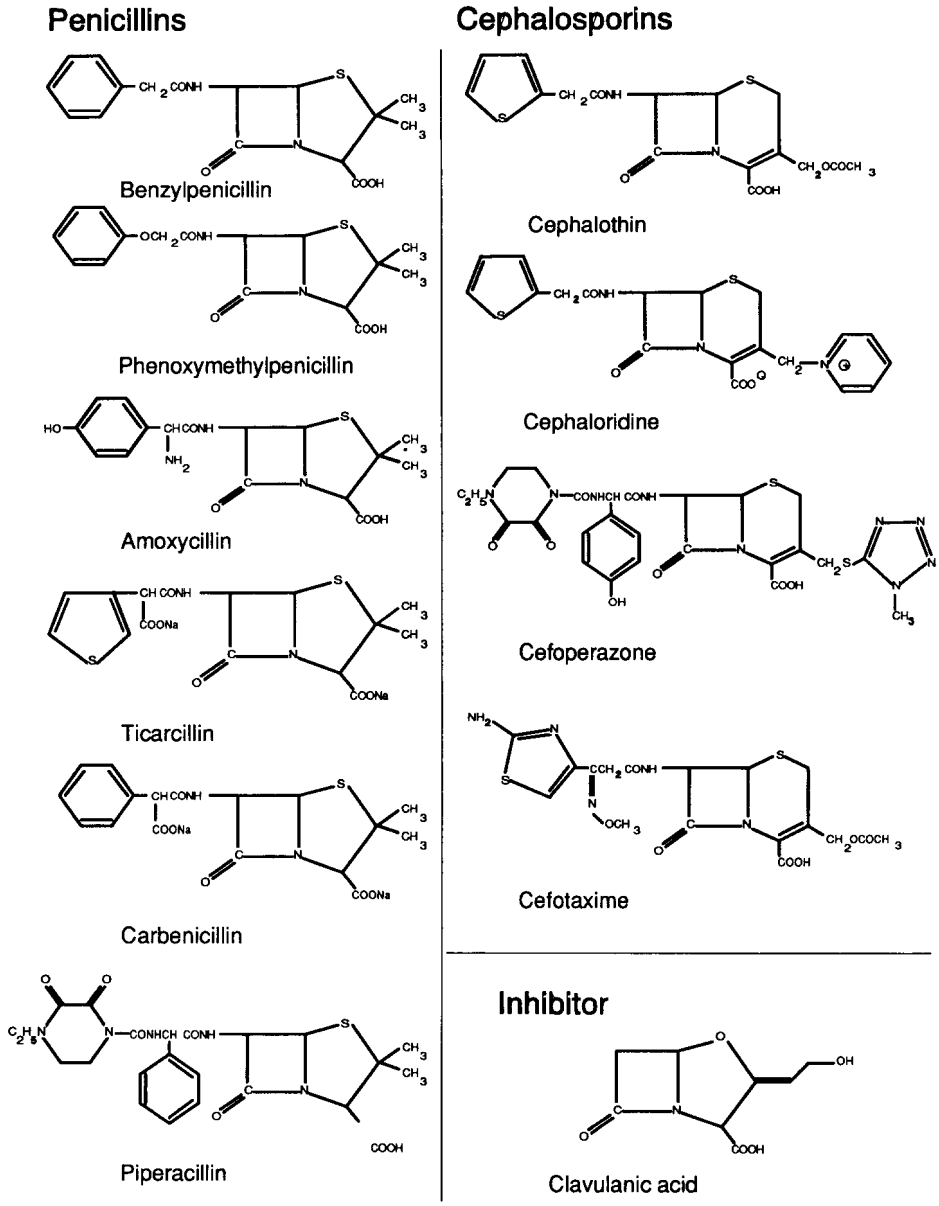

FIG. 1. **β-Lactam antibiotics used in this study.**

context of the amber codon to be suppressed, thus resulting in variable amounts of the protein being made. However, previous measures (11) showed that as little as 1/10,000 of the activity of the wild type is readily detected with the antibiotic disc assays and actually still confers resistance to the selected penicillin. In the case of the aforementioned substitutions, one can assume from the inhibition diameters that the residual activity of these protein variants does not fall even within that range.

Of the 14 protein variants obtained at position 234, only three enzymes were significantly active: the ones obtained with the lysine, arginine, or threonine inserting suppressor tRNAs. Unfortunately, the threonine suppressor inserts both threonine and lysine (17), thus making the corresponding results in Table I inconclusive. To study further these substitutions, the amber mutation was reverted by oligonucleotide-directed mutagenesis, to the missense codons CGG and ACG for arginine and threonine, respectively. The pUC *bla* promoter was then replaced by the stronger promoter from T284. No difference in the level of expression between K234R, K234T β-lactamase, and the wild type enzyme was observed by immunoblots. The antibiotic disc assays (Table II) did not reveal major differences for the protein variant K234R compared with the wild type. This is not the case for the protein variant K234T; the inhibition diameters show a relative decrease in ticarcillin resistance and a totally susceptible phenotype toward cephalosporins. Moreover, the inhibition diameter for ticarcillin combined with clavulanic acid (timentin) was surprisingly small compared with the value for ticarcillin alone.

These first results suggest that these protein variants had enough activity to be analyzed in more detail. The enzymes were then purified almost to homogeneity, as described under "Materials and Methods."

The Mutant Enzyme K234R

Kinetic Parameters—Detailed kinetic studies were then performed using the microacidometric method on a large variety of substrates: amino-, carboxy-, and ureidopenicillins and first and third generation cephalosporins. The kinetic constants for the enzymes are given in Table III.

The k_{cat} values for K234R and TEM-1 β-lactamases for

benzylpenicillin are similar. However, the mutation K234R causes significant changes in the catalytic profile toward the other penicillins. The mutant enzyme presents a greater ability to hydrolyze the carboxypenicillins and the ureidopenicillin tested (6–20-fold increase). In contrast, the affinity has been decreased 10–20-fold for all the penicillins, including the carboxypenicillins. Thus, the mutant K234R exhibits significant decrease in catalytic efficiency (k_{cat}/K_m) toward benzylpenicillin and aminopenicillins, whereas the catalytic efficiency toward carboxypenicillin is conserved. In constrast, the hydrolysis of all the cephalosporins tested is unmodified by the mutation. Both the k_{cat} and the affinity constant K_m remain similar for the K234R mutant and TEM-1 enzyme.

TABLE I

Values of inhibition diameters given by antibiotic disk assays for the XaC-1 strain and the 14 suppressor strains expressing the wild type enzyme or the β-lactamase mutant gene K234am

In the first column, we reported the amino acid inserted by suppression. The values of the inhibition diameters are given in millimeters; R, no inhibition zone (*i.e.* diameter < 7); AMX, amoxycillin; AMC, augmentin (amoxycillin + clavulanic acid); TIC, ticarcillin; TCC, timentin (ticarcillin + clavulanic acid); CF, cephalothin; CFP, cefoperazone; and CTX, cefotaxime.

Amino acid inserted by the suppressor	AMX	AMC	TIC	TCC	CF	CFP	CTX
Ala	19	22	25	31	20	31	34
Arg	**R**	24	16	32	21	31	34
Cys	12	24	21	33	18	31	35
Gln	15	21	18	26	20	32	35
Glu	11	25	25	33	20	32	35
Gly	12	25	14	32	20	30	35
His	16	25	18	33	21	32	35
Leu	20	25	28	33	19	32	40
Lys	**R**	23	**R**	28	20	28	34
Phe	14	25	25	33	21	32	35
Pro	19	26	25	31	21	30	35
Ser	9	25	14	33	23	31	38
Thr	**R**	22	**R**	27	19	26	34
Tyr	12	25	14	33	22	31	35
K234am	19	25	28	33	20	31	36
TEM-1	R	12	R	14	R	R	35

TABLE II

Antibiogram disk assays for the protein variants K234R and K234T compared with the wild type enzyme

The diameters are given in millimeters; R, no inhibition zone (*i.e.* diameter < 7). Other abbreviations are defined in Table I legend.

	AMX	AMC	TIC	TCC	CF	CFP	CTX
K234R	R	10	R	12	9	R	34
K234T	R	11	12	14	19	29	34
TEM-1	R	10	R	14	R	R	34
K234am	15	25	28	33	22	31	34

Interactions with Clavulanic Acid—Clavulanic acid is the first inhibitor effective *in vitro* as well as *in vivo* when combined with amoxicillin or ticarcillin. The competitive and irreversible inhibition can be fitted to Scheme 1 (31, 32):

$$E + I \xrightarrow{k_1} (E, I) \xrightarrow{k_2} (E, I)^* \xrightarrow{k_4} E - I$$
$$\searrow^{k_3} \quad E + P$$

SCHEME 1.

where (E,I) is the noncovalent Michaelis complex, $(EI)^*$ the acyl-enzyme, $(E - I)$ an inactivated form of the enzyme still covalently linked to the inhibitor, and P a degradation product of the clavulanic acid. The different parameters relative to the inhibition are reported in Table IV. The ratio k_3/k_4 (or turnover number, t_n) represents the number of molecules metabolized before the (EI^*) complex is rearranged into its inactivated form. Therefore, a variation of the k_3/k_4 ratio can be explained by a variation of the deacylation rate constant k_3 or by an opposite variation in the value of k_4. Starting from the expression of the rate of inactivation in terms of the instantaneous concentration of EI^* given by Equation 1.

$$d[E - I]/dt = k_4\,[EI^*] \quad (1)$$

The rate of formation of EI^* is then given by Equation 2.

$$d[EI^*]/dt = k_2[E,I] - k_3[EI^*] - k_4[EI^*]$$
$$= k_2[E,I] - (k_3 + k_4)[EI^*] \quad (2)$$

Applying Bodensteins's steady state approximation, which in the context of Equation 2 sets $d[EI^*]/dt = 0$, we obtain

$$0 = k_2[E,I] - (k_3 + k_4)[EI^*] \quad (3)$$

which can be rearranged into

$$[EI^*] = [E,I]k_2/(k_3 + k_4). \quad (4)$$

Substituting Equation 4 into Equation 1, we find that

$$d[E - I]/dt = [E,I]k_2 \cdot k_4/(k_3 + k_4). \quad (5)$$

Since $[E,I]$ is the instantaneous concentration of the non-

TABLE III

Kinetic constants of the protein variants K234R and K234T compared with the wild type enzyme TEM-1

The k_{cat} and the K_M values were determined as described under "Materials and Methods." The k_{cat}/K_M were calculated. ND, not determined. Asterisks indicate that the values could not be determined.

	TEM-1			K234R			K234T		
	k_{cat}	KM	k_{cat}/KM	k_{cat}	KM	k_{cat}/KM	k_{cat}	KM	k_{cat}/KM
	s^{-1}	μM	$M^{-1}\,s^{-1}$	s^{-1}	μM	$M^{-1}\,s^{-1}$	s^{-1}	μM	$M^{-1}\,s^{-1}$
Penicillins									
Benzylpenicillin	1200	24	$5.0 \cdot 10^7$	1000	240	$4.2 \cdot 10^6$	25	900	$2.7 \cdot 10^4$
Penicillin V[a]	1140	25	$4.5 \cdot 10^7$	500	100	$5 \cdot 10^6$	ND	ND	ND
Amoxycillin	1008	43	$2.3 \cdot 10^7$	600	155	$3.85 \cdot 10^6$	7.5	>1000	$<7.3 \cdot 10^3$
Ticarcillin	36	10	$3.6 \cdot 10^6$	600	240	$2.5 \cdot 10^6$	1.125	>1000	$<1.1 \cdot 10^3$
Carbenicillin	120	14	$8.5 \cdot 10^6$	700	180	$3.8 \cdot 10^6$	ND	ND	ND
Piperacillin	144	260	$5.5 \cdot 10^5$	950	>250	$<3.8 \cdot 10^6$	ND	ND	ND
Cephalosporins									
Cephalothin	144	350	$4.1 \cdot 10^5$	90	250	$3.6 \cdot 10^5$	<0.5	**	**
Cephaloridine	2040	612	$3.3 \cdot 10^6$	1700	800	$2.1 \cdot 10^6$	ND	ND	ND
Cefoperazone	492	240	$2.1 \cdot 10^6$	800	600	$1.3 \cdot 10^6$	<0.5	**	**
Cefotaxime	3.6	ND	ND	1	ND	**	**	**	**

[a] Phenoxymethylpenicillin.

TABLE IV
Kinetic constants of the TEM-1, K234R, and K234T for clavulanic acid

	K_i	k_3/k_4	k_{inac}
	μM		s^{-1}
TEM-1	0.08	100	0.02
K234R	0.5	30	0.032
K234T	300	300,000	<0.00019

covalent Michaelis complex, the factor $k_2 \cdot k_4/(k_3 + k_4)$ is the rate constant for inactivation of the enzyme, on the assumption of the irreversible reaction Scheme 1. This inactivation rate constant, k_{inac}, can be approximated to $k_2 \cdot k_4/k_3$, as $k_4 \ll k_3$. The turnover number can then be correlated with the k_{cat}/k_{inac} ratio.

We observed some difference in the interaction with clavulanic acid between the mutant K234R and TEM-1. The affinity of K234R for clavulanic acid is reduced 5–8-fold, in the same proportion as its affinity for penicillins. Nevertheless, the inactivation constant k_{inac} is slightly increased, the inactivation then becomes a little more efficient. As the catalytic constant k_{cat} is lower, this behavior could explain in part the decrease of the turnover number. This decrease of the turnover is probably due to a decrease of the deacylation rate constant k_3 more than k_4.

In the mutated enzyme K234R, the reversible formation of the enzyme-inhibitor complex characterized by the affinity constant K_i is reduced compared with the wild type, whereas the rearrangement into the inactivated complex appears to be more efficient.

Effects of pH on Kinetics Properties—Replacement of lysine 234 by an arginine in β-lactamase TEM-1 does not modify the isoelectric point of the enzyme, and the titration curve does not reveal a difference in charge, over the pH range 3–9 (data not shown). This is not so surprising, as both the lysine and the arginine are protonated in this range of pH. Nevertheless, we studied the pH dependence of the kinetic constants for benzylpenicillin and cephalothin of the K234R mutant compared with the wild type.

The plots for k_{cat} against pH over the range 5.5–8.5 for hydrolysis of benzylpenicillin and cephalothin are reported in Fig. 2. The optimum pH for hydrolysis of both substrates by K234R is one unit lower than that of the wild type. For the K_m values, which are plotted in Fig. 3, there is a clear dependence on the pH. The affinity is conserved at low pH range (pH < 6.5) and at higher pH, the constant K_m increases with pH both for benzylpenicillin and cephalothin. Although the affinity constants differ by one order of magnitude, the K_m values for benzylpenicillin for K234R and the wild type follow the same profiles, and the discrepancy between the curves remains the same over the pH range. For cephalothin, the K_m curves present essentially the same pattern, with a slightly better affinity for the mutant K234R than the wild type. This behavior corroborates the k_{cat} values (Table III), which showed that the hydrolysis of penicillins is more perturbed than that of cephalosporins with the mutant enzyme K234R. The guanidine function of arginine, with its side chain 1 Å longer, is probably in a spatial location which still remains correct for the hydrolysis of cephalosporins.

The curve for k_{cat}/K_m (Fig. 4) for K234R compared with the wild type shows that the apparent alkaline pK_2 for the free enzyme was unmodified. These indications strongly suggest that the lysine is not directly responsible for the pH dependence of k_{cat}/K_m.

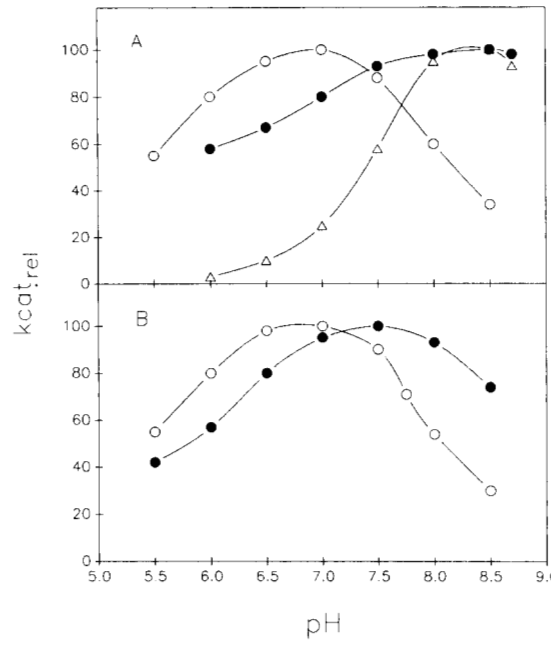

FIG. 2. **pH dependence of the relative k_{cat} for wild type (○), K234R (●), and K234T (△) enzymes.** The hydrolysis of benzylpenicillin (*A*) or cephalothin (*B*) was measured at 37 °C by computerized microacidometry using [S] ≫ K_m. The *ordinate* represents the rate constant k_{cat} relative to its maximum value over the pH range tested.

The Mutant Enzyme K234T

Kinetic Parameters—The conversion of lysine 234 to a threonine had a drastic effect on activity toward penicillins. The k_{cat} for benzylpenicillin was reduced about 50-fold, and the K_m was increased by a comparable amount (K_m = 900 μM). For the other penicillins, the affinities were so drastically decreased that the K_m values cannot be measured. Thus, the catalytic efficiency k_{cat}/K_m was very low (1000-fold reduction). Moreover, the k_{cat} values for all the cephalosporins dropped 300–1000-fold compared with the wild type enzyme. Consequently, the mutation resulted in more than 99.7% reduction of its activity (Table III) toward cephalosporins, but the enzyme still retained a significant activity toward penicillins.

Interactions with Clavulanic Acid—The suicide inhibitor clavulanic acid was also tested on the mutant K234T. In constrast to the mutant K234R, K234T presents an original behavior toward clavulanic acid. The K_i constant and the turnover number were respectively 3000- and 300-fold higher than for the TEM-1 enzyme. This mutant seemed unable to bind the clavulanic acid in the active site; the interactions became unspecific. The k_{inac} was similarly reduced (approximately 1000-fold). This indicates that this mutation results in a great reduction in efficiency of inactivation. The entire inactivation process is altered.

Effects of pH on Kinetics Properties—The pI value for K234T is 5.35. Moreover, the titration curves (data not shown) reveal a small difference of charge at pH > pI between K234T and RTEM-1 and none between K234R and the wild type. This small decrease in the isoelectric point for the mutant K234T was expected based on the change from the positively charged ammonium group of lysine 234 to the uncharged group of the threonine.

Interestingly, the mutant K234T kept the same optimum pH (Fig. 2*A*), but it was essentially inactive at pH = 6 and reached 10% of the wild type activity at pH = 8. The basic environment at pH = 8 therefore enhanced the catalytic activity.

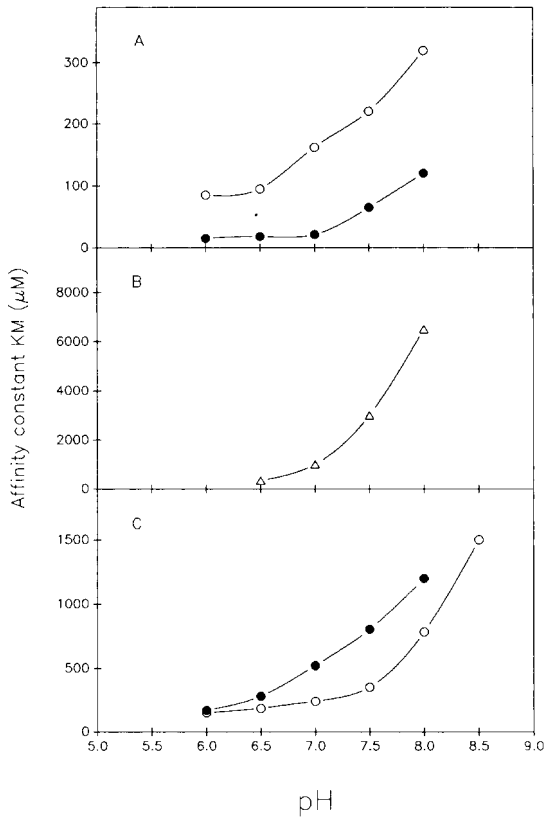

FIG. 3. **pH dependence of the affinity constant K_m for wild type (○), K234R (●), and K234T (△) enzymes.** The affinity constant K_m toward benzylpenicillin (*A* and *B*) and cephalothin (*C*) was measured at 37 °C by computerized microacidometry.

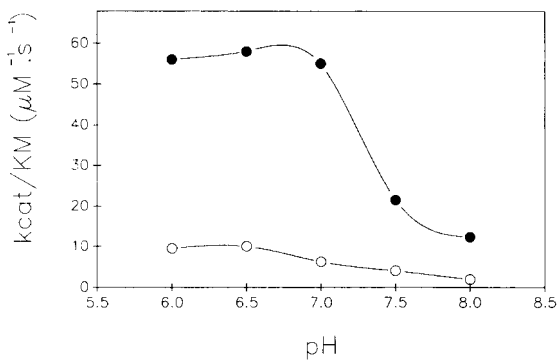

FIG. 4. **pH dependence of the catalytic efficiency (k_{cat}/K_m) for wild type (○) and K234R (●) enzymes.** This plot shows the k_{cat}/K_m ratio for benzylpenicillin and gives apparent alkaline pK_2 values of 7.4 for both free enzymes.

The plot for K_m over the pH range (Fig. 3*B*) presents the same profile as for TEM-1, but values are 3 orders of magnitude higher.

DISCUSSION

Systematic amino acid replacement of lysine 234 in β-lactamase TEM-1 with nonsense suppressors enabled us to select quickly two mutants with interesting enzymatic activity: K234R and K234T. Kinetic study of these mutants yields valuable information on the role of this conserved residue in the active site box VII.

The mutant K234R compared with the wild type exhibits a similar catalytic constant k_{cat} for penicillin G. However, the catalytic efficiency k_{cat}/K_m toward penicillins was modified, as the mutant showed close to 10-fold increase in K_m for the penicillins. Both the amine function of the lysine or the guanidine function of the arginine are highly basic with respective pK_a values of 10.53 and 12.48. In the range of pH values compatible with a good stability for the enzymes and the β-lactams (*i.e.* 5–9), both residues are fully protonated. Nevertheless, arginine is more bulky than lysine, and the increase in side-chain length can be estimated to about 1 Å. The resulting steric hindrance could be responsible for the increase in K_m for penicillins.

Comparison of three-dimensional structures of various β-lactam antibiotics (33) showed that the distance between the oxygen atom of the amide group and the carbon of the carboxylic group of "active" β-lactams is in the range of 3.0–3.9 Å. This distance is 3.9 Å for ampicillin and 3.2 Å for cephaloridine, which makes a 0.7-Å difference. In relation to the position of the β-lactam ring in the active site of β-lactamases, the carboxylic groups are not in the same spatial location for penicillins and cephalosporins. If the postulated salt bridge between the C3 carboxylic group of penicillins and the amine function of the lysine is disrupted in the mutant β-lactamase K234R, this cannot be the case with cephalosporins where the C4 carboxylic group probably cannot make such an ionic interaction with the wild type protein.

This last hypothesis is also supported by work from Laws and Page (34), who have compared the second order rate constants for the hydrolysis of esters and lactones of penicillins and cephalosporins catalyzed by *Bacillus cereus* I and II β-lactamases. They have shown that the hydrolysis is 50 times better for a cephalosporin lactone than for an analogous cephalosporin. It appears that the two oxygen atoms of the lactone in the cephalosporin lactone carry considerable negative charge and could interact with a suitably placed positive charge such as lysine 234.

The results obtained with the mutant K234T, in which the basic group is removed, support the importance of the binding of the carboxyl group of the penicillins with the basic lysine residue 234 for the positioning of the substrate in the active site cavity. This modified enzyme exhibited a very low affinity toward penicillins, representative of a severe decrease of the binding of the substrates in the active site, although it retained a noticeable hydrolysis capability toward most penicillins. This activity varied largely with pH; the residual activity for mutant K234T represents 2% of that of the wild type at pH = 7 and shifted to 10% at higher pH. In contrast, the removal of the basic group was critical for the activity toward cephalosporins; the enzyme lost >99.7% of its activity toward these substrates. This mutant K234T can be compared with the mutated *Bacillus* enzymes K234E and K234A studied by Ellerby *et al.* (13).

On the other hand, this mutant presents a very interesting point; its susceptibility to clavulanic acid is so reduced that we can consider that this compound is no longer an inhibitor of the enzyme. The kinetic constants indicate that the clavulanic acid reacts very slowly with the K234T protein variant. Thus, the inactivation is no more effective although most penicillins can still be hydrolyzed. On the contrary, we have shown that inhibition of the K234R mutant by clavulanic acid is barely modified, compared with the wild type. The inactivation of β-lactamase TEM-1 or K234R mutant by clavulanic acid is rapid and effective. This inhibitor presents structural similarities to penicillins, with steric hindrance largely reduced on account of the lack of lateral chain substitutions on the 6-aminopenicillanic acid nucleus. So, the drastic reduction in inactivation efficiency for the mutant enzyme K234T is essentially due to the basic group removal. Conse-

quently, the basic group appears to be essential for the recognition and binding of inhibitors and substrates in the active site.

Moreover, the mutation Lys-234 → Arg extends the substrate profile of the enzyme to carboxypenicillins. An amino acid sequence comparison of the class A carbenicillin-hydrolyzing enzymes or CARBs (14) shows that these enzymes contain an unusual arginine instead of a lysine in the Lys-Ser-Gly box. This was shown for most of these enzymes: PSE-4 (35), CARB-3, and CARB-4 (14), but not the PSE-3 enzyme (36). The high hydrolytic activity of these enzymes for carbenicillin, ticarcillin, and related compounds, which have been characterized previously (37), could be in part correlated with the presence of the arginine at position 234. These penicillins possess, on the 6-aminopenicillanic acid nucleus, a side chain substitued by a carboxylic acid. This strongly suggests that the basic guanidium group of the arginine residue, in contrast to the amine group of the lysine, places this side chain in a spatial location where new interactions are favorable.

Nevertheless, although the mutant K234R exhibits a high hydrolytic activity toward carbenicillin and ticarcillin, the affinity constant remains very high. The sequence alignments of the TEM and SHV enzymes with the carbenicillin-hydrolyzing enzymes suggest that the carbenicillinase character could be associated with two events: the presence of an arginine at the Lys-Ser-Gly box, as we have just seen and a glycine, found in all four carbenicillinases at position 240, where there is usually an aspartic or a glutamic acid for the TEM and SHV enzymes. The low affinity of K234R can be caused both by a steric hindrance and by an electrostatic repulsion between the lateral chain of carbenicillin or ticarcillin with the acidic residue at position 240. We are just testing this hypothesis by creating the single mutation E240G on the TEM-1 enzyme and on the K234R mutant.

The effects of the mutations on k_{cat} can be correlated with a modification in transition state binding. The difference between the activation free energy of the native and the mutant enzymes can be calculated using the following equation (38).

$$\Delta G_{app} = -RT\ ln[(k_{cat}/K_m)mut/(k_{cat}/K_m)wt] \quad (6)$$

Comparison of the ratios k_{cat}/K_m for wild type and mutants for benzylpenicillin gives the apparent contribution of the side chain of the different amino acids to the binding energy of the enzyme transition state complexes. The transition state energy increased by 1.4 kcal/mol for K234R and 4.6 kcal/mol for K234T. The first value fits with a deletion of a side chain that forms a good hydrogen bond with an uncharged group, while the second value fits with a deletion of a side chain that forms an ionic bond (38, 39). According to the crystallographic data for the *B. licheniformis* β-lactamase (5), we postulate that in the first case, the mutant K234R lost the H-bond between serine 130 and lysine 234 which plays a role to maintain the geometry of the active site, but the arginine maintains the electrostatic environment for the substrate binding. In the second case, both the aforementioned hydrogen bond and the ionic interaction of the basic group of the enzyme with the carboxylate of the substrate are inexistant.

The variation of k_{cat} values over the pH range was unexpected, since both lysine and arginine are protonated at the pH where the measures of kinetic constants were done. Such a paradoxical result was also obtained by Ellerby *et al.* (13) for their K234E mutant. Moreover Anderson and Pratt (40) have shown that K_S and the rate constants k_2 and k_3 vary differently according to pH. The modification of the K_m value clearly suggests that some of or all of these parameters, k_2, k_1, k_{-1}, are perturbed by the replacement of lysine to arginine or lysine to threonine. One can only tentatively conclude that the observed behavior results from several perturbations of the different existing bonds in the active site, although lysine 234 does not seem to be directly implied in the proton transfer during hydrolysis (since pK_2 remains unmodified). This assumption is supported by the variations of the activation free energy.

In conclusion, for a broad spectrum β-lactamase like TEM-1, single amino acid substitutions reveal the complexity of the interaction between the enzyme and its various substrates. Replacing the lysine with an arginine enhances transition state binding for carboxypenicillins, whereas it does not change that of cephalosporins and slightly decreases k_{cat} for penicillins. At the same time, K_m for cephalosporins is unaffected, whereas it is increased for penicillins, including carboxypenicillins. Thus, increasing the size of the charged side chain at position 234 affects both binding and catalysis for penicillins, while it does not affect cephalosporins. Removing the positive charge, on the other hand, results in drastic loss of activity toward all β-lactams, especially cephalosporins, affecting both K_m and k_{cat}. The electrostatic environment provided by lysine 234 plays a major role in transition state stabilization, mainly as an electrostatic anchor between the basic group and the carboxylate of substrates.

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